

Fall 12-17-2014

Purification of Soluble Recombinant Salmonella typhimurium Flagellin (FliC) Protein Constructs Expressed in Escherichia coli

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Georgia State University

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PURIFICATION OF SOLUBLE RECOMBINANT *SALMONELLA TYPHIMURIUM* FLAGELLIN
(FliC) PROTEIN CONSTRUCTS EXPRESSED IN *ESCHERICHIA COLI*

by

JENNIFER ANN HOOKER

Under the Direction of George E. Pierce

ABSTRACT

A platform for vaccine development has been developed at Georgia State University utilizing recombinant *Salmonella typhimurium* flagellin (FliC) fused to an antigen that can be overexpressed in *Escherichia coli* grown in a two-stage fermentation. The flagellin acts as an adjuvant to increase the immunopotency of the fused antigen. Flagellin is the ligand for Toll-like Receptor 5 (TLR5), a part of the innate immune system. Binding of the flagellin:antigen recombinant protein to TLR5 triggers a strong innate and adaptive immune response to the fused antigen leading to a potentially strong protective immunity to the antigen.

Purification of the recombinant FliC fusion protein must meet rigorous criteria in order to be used as a vaccine. One of the major issues in purifying recombinant proteins expressed in a Gram-negative bacterium is the removal of endotoxin. Small amounts of

endotoxin present in a vaccine can lead to serious complications, including death.

Recombinant proteins are also expressed as either soluble or insoluble protein when over expressed in *E. coli*. Soluble proteins expressed by the bacterium are properly folded and biologically active, however removal of contaminants such as endotoxin, can be problematic. Insoluble protein is improperly folded and biologically inactive. The insoluble proteins aggregate into inclusion bodies with little or no contaminants associated with the protein, making purification easier. However, in order to restore the biological activity of the insoluble protein, it must first be solubilized and then refolded. This process is often expensive and time consuming, as there is currently no standardized method for protein refolding.

In this study a purification method for the soluble protein of two FliC constructs, full-length FliC and FliC fused to a Marburg virus antigen, was evaluated for effectiveness in purification, removal of endotoxin and maintaining TLR5 activity. The proteins of interest were purified utilizing only the soluble protein containing the properly folded and biologically active recombinant protein. Utilizing methods for purification that take advantage of physical and chemical properties of the protein the recombinant proteins were purified and the level of endotoxin reduced to levels acceptable for use as a vaccine. The TLR5 activity of the soluble recombinant proteins was compared to recombinant protein that had been purified using a denaturing and refolding step. The soluble protein elicited a higher TLR5 response at a lower concentration of protein than the refolded protein. Purification of the soluble fraction also involved fewer step and less time than purification of both the soluble and insoluble protein.

INDEX WORDS: Flagellin, FliC, Toll-like Receptor 5, Soluble Protein Purification, Recombinant Protein

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JENNIFER ANN HOOKER

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

in the College of Arts and Sciences

Georgia State University

2014

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2014

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JENNIFER ANN HOOKER

Committee Chair: George E. Pierce

Committee: Sidney Crow Jr.

Eric Gilbert

Electronic Version Approved:

Office of Graduate Studies

College of Arts and Sciences

Georgia State University

December 2014

DEDICATION

I would like to dedicate this to my children, Anna and Jack and especially my husband, John. Without their support, encouragement and understanding, none of this would have been possible. I love you more than words can express.

ACKNOWLEDGEMENTS

I would like to thank Dr. George Pierce for his patience and direction on my project. I would also like to thank Dr. Sidney Crow and Dr. Eric Gilbert for their guidance and for being on my committee. I would like to thank the members of the Pierce lab for their support and help throughout this process. Very special thanks to Dr. Trudy Tucker and Dr. Shelby Jones for their support and encouragement and to Dr. Feng Du for always being willing to help and offer suggestions. I would also like to thank Dr. Julia Hilliard for allowing me to use her lab for tissue culture work and Dr. Mugdha Vasireddi for her assistance in setting up the HEK cells and taking the time to show me how to maintain them. Thanks also to Dr. Yan Chen and John Mischak of VaxInnate for their help and input. Last, but not least, to my husband, John Hooker, whose unconditional love and support allowed me to complete this project.

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LIST OF ABBREVIATIONS

HPV.....	Human Papilloma Virus
GSU.....	Georgia State University
APC.....	Antigen Presenting Cells
TLR.....	Toll-like Receptor
IL.....	Interleukin
TIR.....	Toll-interleukin receptor
LRR.....	Leucine rich repeat
PAMPs.....	Pathogen-Associated Molecular Patterns
TLR5.....	Toll-like Receptor 5
DCs.....	Dendritic Cells
FA4000.....	Recombinant Full- Length FliC Flagellin
5088.....	Recombinant FliC:GP132 Fusion Protein
ET.....	Endotoxin
LPS.....	Lipopolysaccharide

EU.....	Endotoxin Units
IB.....	Inclusion Bodies
PEG 3350.....	Polyethylene Glycol 3350
PEG.....	Polyethylene Glycol
TFF.....	Tangential Flow Filtration
AEX.....	Anion Exchange Chromatography
HIC.....	Hydrophobic Interaction Chromatography
HEK.....	Human Embryonic Kidney
hTLR5 cells.....	HEK-Blue™ hTLR5 Cells
hTLR5.....	Human TLR5
SEAP.....	Secreted Embryonic Alkaline Phosphatase
null cells.....	HEK-Blue™ null cells
RL.....	Refined Lysate
AP.....	Aqueous Phase

DP.....	Detergent Phase
PES.....	Polyethersulfone
FAP.....	Filtered Aqueous Phase
NaCl.....	Sodium Chloride
CV.....	Column Volume
LAL.....	Limulus Amebocyte Lysate
CSE.....	Control Standard Endotoxin
PBS.....	Phosphate Buffered Saline
TBST.....	Tris-Buffered Saline with 1% Tween20
HRP.....	Horse Radish Peroxidase

1. INTRODUCTION

Conventional vaccines currently licensed for use in the United States typically consist of: 1) attenuated whole cells 2) inactivated whole cells or 3) a purified antigenic component of the microorganism. While vaccines presently in use have been successful against a variety of pathogens, most require multiple doses to confer lasting immunity and there are many diseases for which there is no vaccine available. Many vaccines or vaccine candidates do not elicit a strong enough immune response when given alone to be effective requiring the use of an adjuvant to increase the potency of the vaccine. The number of adjuvants currently approved for use in vaccines is extremely limited due to side effects that can be caused by the adjuvant and the stringent criteria required for licensing. Currently, there are only two types of adjuvants licensed for use in vaccines in the United States: alum, which is composed of aluminum salt or gel and AS04 (a combination of aluminum hydroxide and monophosphoryl lipid A), which is only used in the Human Papilloma virus (HPV) vaccine. Production of some vaccines can also be time consuming and may not yield enough vaccine to meet demand. For example, influenza vaccine is currently grown in eggs, with a production time of 6-9 months and each egg yielding only 1-3 doses of vaccine. Under the current method of production, it would be difficult to redirect the production process for a pandemic or variant seasonal strain of influenza. An ideal vaccine would be one that could be produced quickly and inexpensively, confers lasting immunity and requires a single dose (Levine and Stein, 2004; Zinkernagel, 2003). Advances in the understanding of the immune system response and genetic engineering have led to new approaches in vaccine development. At Georgia State University (GSU), a vaccine platform utilizing recombinant flagellin fused to a bacterial or viral antigen has

been developed that can be efficiently overexpressed in *E. coli* grown in a large scale fermentation and purified. The flagellin acts as an adjuvant significantly increasing the immunopotency of the linked antigen (Huleatt et al., 2007; McDonald et al., 2008).

The human immune system is made up of two components: the innate immune system and adaptive immune system. For a vaccine to confer lasting immunity, it must induce a strong immune response in both the innate and adaptive immune systems. The innate immune system comprises the “first response” to an infectious agent. Once a pathogen is detected, the innate immune system launches a non-specific response utilizing interferon, complement, natural killer cells and activated phagocytes in an attempt to clear the infection. Antigen presenting cells (APCs), a component of the innate immune system, link the innate and adaptive immune system. Antigen from the pathogen is processed by the APCs and presented to the adaptive immune system that is composed of B- and T- cells. Once presented with antigen, the B- and T-cells become activated. Activated B-cells secrete antibodies that are specific to the antigen and T-cells directly attack the pathogen to clear the infection. A subset of these activated B- and T- cells become “memory” cells. When presented with an antigen the adaptive immune system has previously been exposed to, the immune system is able to launch an antigen specific response using these memory cells, resulting in a faster and more effective immune response. An effective vaccine must create protective immunity by inducing production of memory cells (immunological memory), a process that is in part activated by the innate immune response.

The innate immune systems of both vertebrates and invertebrates have developed a strategy to detect microbial infection using Toll-like receptors (TLRs) that recognize highly conserved regions of pathogens that are unique to microorganisms. TLRs are a member of

the Interleukin-1 receptor/Toll-like receptor superfamily based upon homology of the Toll-interleukin (IL)-1 receptor domain (TIR). TLRs are trans-membrane proteins that function as homo- or hetero-dimers with three functional domains: an extra-cellular leucine-rich repeat (LRR), a membrane spanning portion and an intracellular TIR. The extracellular LRR forms a horseshoe shape that is believed to function in the recognition of a specific microbial motif. The TIR is required for activation of the signal cascade leading to an innate immune response (Akira and Takeda, 2004). Recognition of a specific conserved microbial motif or pathogen-associated molecular pattern (PAMP) by the associated TLR receptor induces a signaling cascade resulting in a non-specific immune response to the pathogen. TLRs have also been shown to play a fundamental role in the induction of the adaptive immune response (Medzhitov et al., 1997; Schnare et al., 2001). There are currently 10 known TLRs expressed in humans that recognize a specific ligand (Table 1). This study focuses on Toll-like receptor 5 (TLR5) and its ligand, flagellin. TLR5 specifically recognizes the protein, bacterial flagellin, and is the only TLR known, to date that exclusively recognizes a single type of protein.

Table 1. Human Toll-like Receptors and their ligands (Akira, 2011; Medzhitov, 2001)

Toll-like Receptor	TLR ligand
TLR1/TLR2	Bacterial lipopeptide
TLR2	Gram-positive lipoproteins, lipomannans and lipotechoic acids
TLR3	Viral double stranded RNA
TLR4	Lipopolysaccharide
TLR5	Bacterial flagellin
TLR6/TLR2	Mycoplasmal macrophage-activating lipopeptide 2kDA(MALP-2)
TLR7	Small synthetic antiviral molecules, single stranded RNA
TLR8	Small synthetic antiviral molecules, single stranded RNA
TLR9	Unmethylated CpG DNA
TLR10	unknown

The TLR5 ligand, flagellin, is the protein monomer that makes up a bacterial flagellum. Each flagellin monomer is composed of four domains: D0, D1, D2 and D3 (Figure 1). The D0 and D1 domains consist of the N-terminal and C-terminal regions and are the most highly conserved regions of flagellin. These two domains make up the flagellar filament backbone and are critical for flagellar filament assembly (Kuwajima, 1998)(Figure 2). A mutation in this region would be detrimental to the microorganism by affecting motility and possibly virulence. Research has shown that a specific region in the conserved D1 domain interacts directly with TLR5 (Eaves-Pyles et al., 2001; Smith et al., 2003). The D2 and D3 region of flagellin comprise the exposed part of the flagellum filament and are highly variable. These regions are responsible for the antigenicity of flagellin (Yoshioka et al., 1995; He et al., 1993). Binding of flagellin to TLR5 induces a MyD88-dependent signaling cascade leading to activation of the transcription factor NF- κ B that in turn activates the innate immune system against a flagellated bacterium and ultimately the adaptive immune system via dendritic cells (Didierlaurent et al., 2004) (Figure 3).

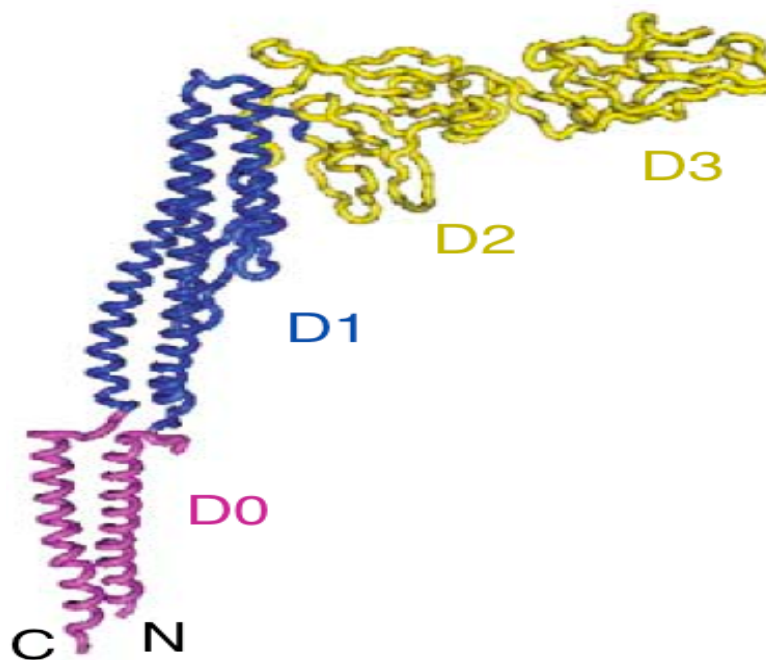


Figure 1. Domains of flagellin monomer, FliC, from *S. typhimurium* (Cruz Ramos, et al, 2004).

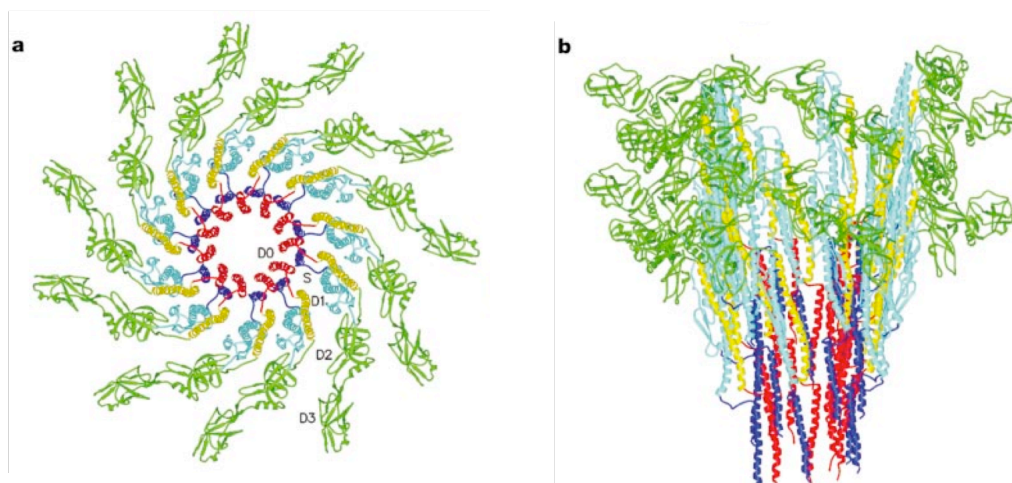


Figure 2. Orientation of flagellin monomer domains in bacterial flagellum. a) flagellum filament as seen from the top b) Flagellum filament viewed from the side (Yonekura, et al, 2003).

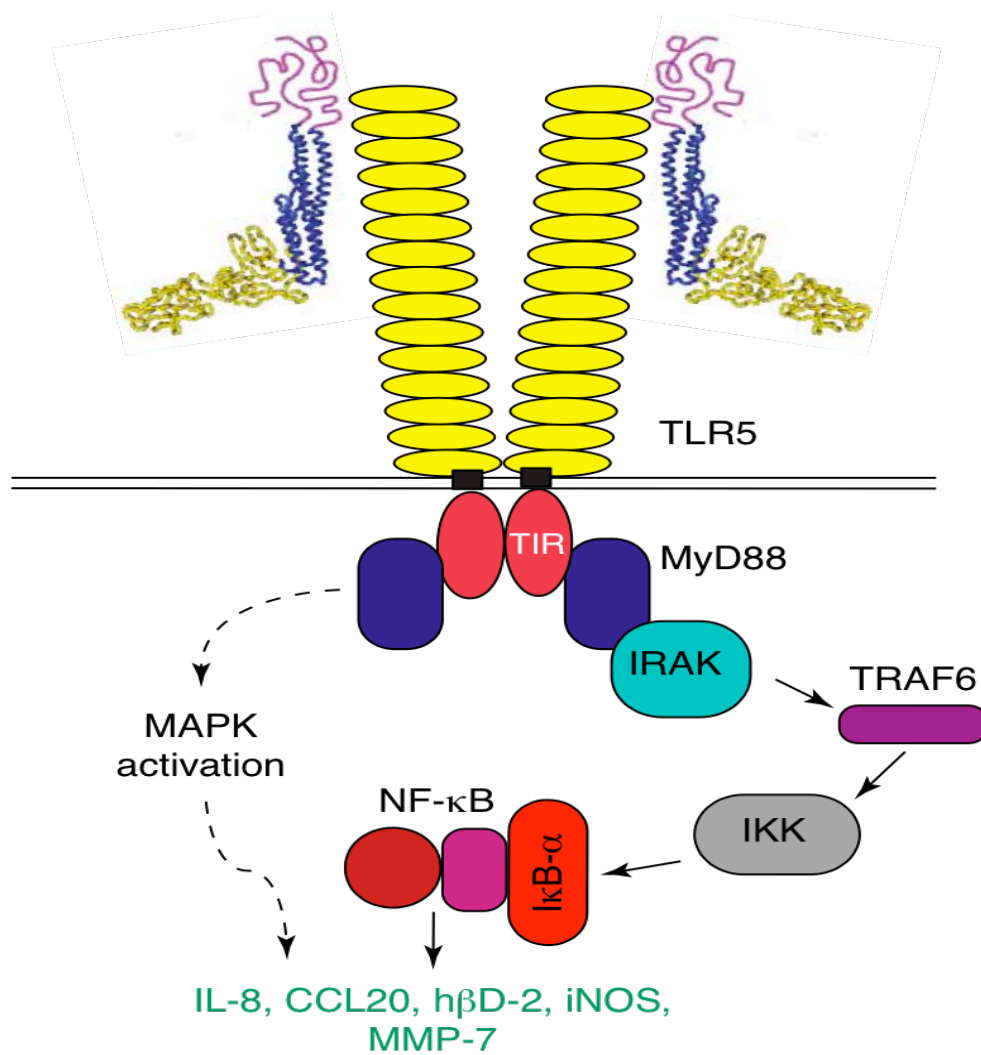


Figure 3. Signaling cascade induced by the binding of flagellin monomer to human Toll-like receptor 5 (Cruz Ramos, et al, 2004).

Toll-like receptor 5 is expressed by dendritic cells (DCs), a class of antigen presenting cells in humans that are present in the nose, lungs, stomach and intestines. Binding of flagellin to the TLR5 receptor present on dendritic cells results in the activation and maturation of the DC. The DC processes the flagellin and the linked antigen and presents both to B- and T- cells present in the lymph nodes, resulting in the activation of both naïve T-cells and B-cells, components of the adaptive immune system (Figure 4). Activation of DCs via TLR5 serves as an important link between the innate and adaptive immune (Iwasaki and Medzhitov, 2004; Means et al., 2003).

The use of flagellin as an adjuvant in vaccines has been shown to greatly increase the immunopotency of an antigen particularly when that antigen is linked to flagellin resulting in activation of both the innate and adaptive immune systems (Huleatt, et al, 2007). By linking an antigen to flagellin, the vaccine takes advantage of a system already present in the innate immune system, inducing a faster and stronger adaptive immune response to an antigen that may have elicited a weak or no immune response. Previous exposure of flagellin to the immune system has not been shown to decrease its effectiveness as an adjuvant allowing it to potentially be used in multiple vaccines and is effective at eliciting a strong immune response at very low doses (Honko et al., 2006; Ben-Yedidia and Arnon, 1998).

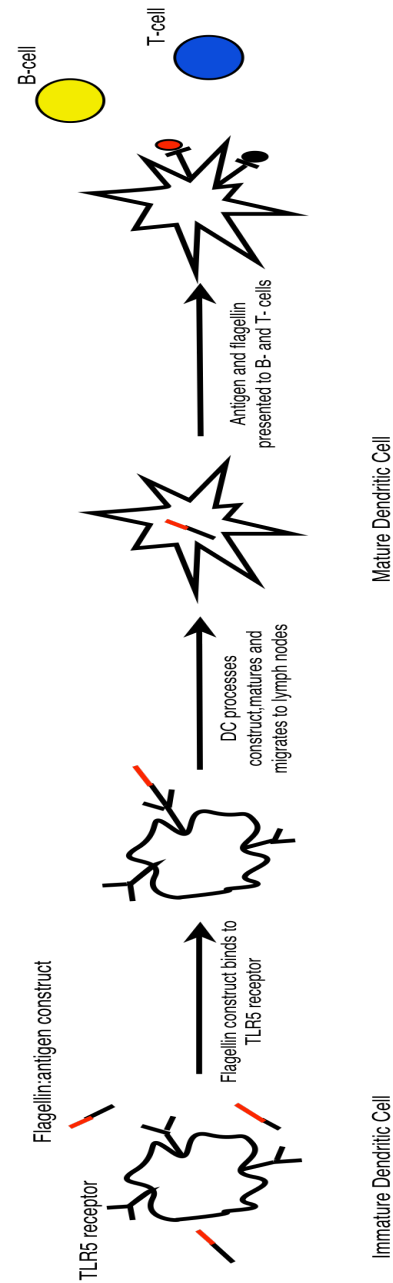


Figure 4. Schematic of dendritic cell activation through binding of flagellin to TLR5 receptor

The recombinant proteins to be discussed were constructed using the DNA sequence for the flagellin protein, *fliC*, from *Salmonella typhimurium* LT2, which was cloned into *Escherichia coli* for expression and protein production. There are several potential insertion sites for antigen in FliC. The insertion sites least likely to affect TLR5 recognition include the N-terminal D0 region, the hypervariable D2-D3 region, and the C-terminal D0 region of FliC, with the latter two being the best potential insertion sites (Figure 5).

Insertion of an antigen into the N-terminal D0 region is possible, specifically amino acids 1-78, but might require a start codon to be engineered into DNA sequence of the antigen.

Research has shown that deletion or insertion of other amino acid sequences into the hypervariable region has had little or no effect on recognition of FliC by TLR5 (Eaves-Pyles et al., 2001). However, the size of the inserted antigen is restricted by the size of the deletion in the D3 region. Insertion of an antigen that is too large for the D3 region might affect the folding of the protein and TLR5 recognition. Because the hypervariable region of FliC is the antigenic region recognized by the human immune system, a benefit of deleting this region is that it would allow the vaccine construct to possibly evade neutralizing antibodies that may decrease the effectiveness of the vaccine (Nempont et al., 2008).

Insertion of an antigen into the N-terminal and C-terminal D1 regions, specifically amino acids 78-129, 135-173 and 395-444, should be avoided. Insertion of antigen into these regions has been shown to disrupt TLR5 recognition of FliC (Smith et al., 2003).

To date, two *S. typhimurium* FliC constructs have been developed and successfully overexpressed in *E. coli* grown in a two-stage fermentation at Georgia State University (GSU), a full-length FliC recombinant protein (FA4000) and a FliC:GP132 recombinant protein (5088) with GP132 fused to the C-terminal end of a FliC flagellin truncated by

approximately 30 amino acids. GP132 is a truncated glycoprotein from Marburg virus that has been shown to induce protective immunity in mice (Kalina, et al, 2009). Expression of recombinant proteins in *E. coli* is a highly effective and inexpensive way to quickly produce large quantities of recombinant protein for use as a vaccine. However, the use of Gram-negative bacteria as a host for recombinant protein expression creates some problems in purification of a protein to be used as a vaccine.

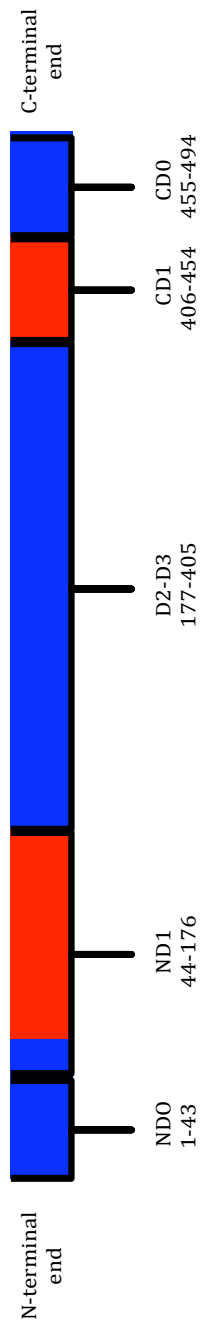


Figure 5. Linearized FliC protein with labeled regions and corresponding amino acid sequence. (ND0- N-terminal D0, ND1 – N-terminal D1, CD1 – C-terminal D1 and CD0 – C-terminal D0.) Blue areas indicates where antigen insertion is possible and red areas indicates where antigen should not be inserted into FliC. (Smith et al, 2003; Samatey et al., 2001 and Yonekura et al., 2003.)

One of the major problems encountered when using a Gram-negative bacterium as a host for expression of a recombinant protein is the presence of significant amounts of endotoxin (ET). Endotoxin is the lipopolysaccharide (LPS) present in the outer membrane of the cell wall in Gram-negative bacteria (Figure 6). ET is a very stable molecule, able to withstand high temperatures and variations in pH. ET also binds to protein with certain proteins having a significantly higher affinity for endotoxin making it difficult to remove from protein preparations. The presence of even a small amount of endotoxin in a recombinant protein vaccine can cause serious side effects such as, a systemic inflammatory reaction that can lead to shock, tissue injury and possibly death. A very small amount of endotoxin is capable of causing side effects, so the removal of endotoxin from protein to be used as a vaccine is crucial (Magalhães et al., 2007). The Federal Drug Administration has established the acceptable endotoxin level in vaccines as 5 endotoxin units (EU)/kg of bodyweight. Additionally, endotoxin has been shown to interfere with a number of *in vitro* assays, including the TLR5 assay used to determine the biological activity of flagellin (Liu et al., 1997).

Another issue stemming from overexpression of recombinant protein in Gram-negative bacteria is how the protein is expressed when expression of a protein is over-induced. Recombinant proteins overexpressed in *E. coli* can be expressed as either soluble or insoluble protein (Figure 6) requiring several different approaches to purification depending on how the protein is expressed in order to optimize the protein yield. Recombinant proteins expressed as soluble protein have a higher percentage of properly folded protein and biologically active molecules, but more contaminants, including endotoxin, which must be removed during purification. Insoluble protein can often

account for over 50% of the expressed protein and these misfolded and/or incompletely folded, inactive proteins aggregate into inclusion bodies (IB). Inclusion body formation is often exacerbated by the over-expression of the recombinant protein in *E. coli* that can lead to an insufficient amount of chaperones in the cell to assist in proper protein folding and competition between protein folding and aggregation (Basu et al., 2011; Georgiou and Valax, 1996). In order to recover the native protein from inclusion bodies, the protein must first be solubilized and then refolded into its proper, biologically active conformation. This step poses a significant problem during purification because there is currently no standardized method for refolding protein and optimal refolding conditions must be determined for each individual recombinant protein construct. The typical yield of bioactive protein following protein refolding is also low, ranging from 15-40%. The low yield of biologically active protein is primarily due to protein aggregation during refolding (Singh and Panda, 2005; Yang et al., 2011). Over all, this process can be extremely time consuming and costly. However, because these proteins are contained in inclusion bodies the level of contaminants, especially endotoxin, is much lower than in the soluble fraction and often requires fewer steps for purification. GSU recombinant FliC protein constructs FA4000 and 5088 expressed primarily as soluble protein during fermentation, with some expression as insoluble protein (Figures 7 and 8).

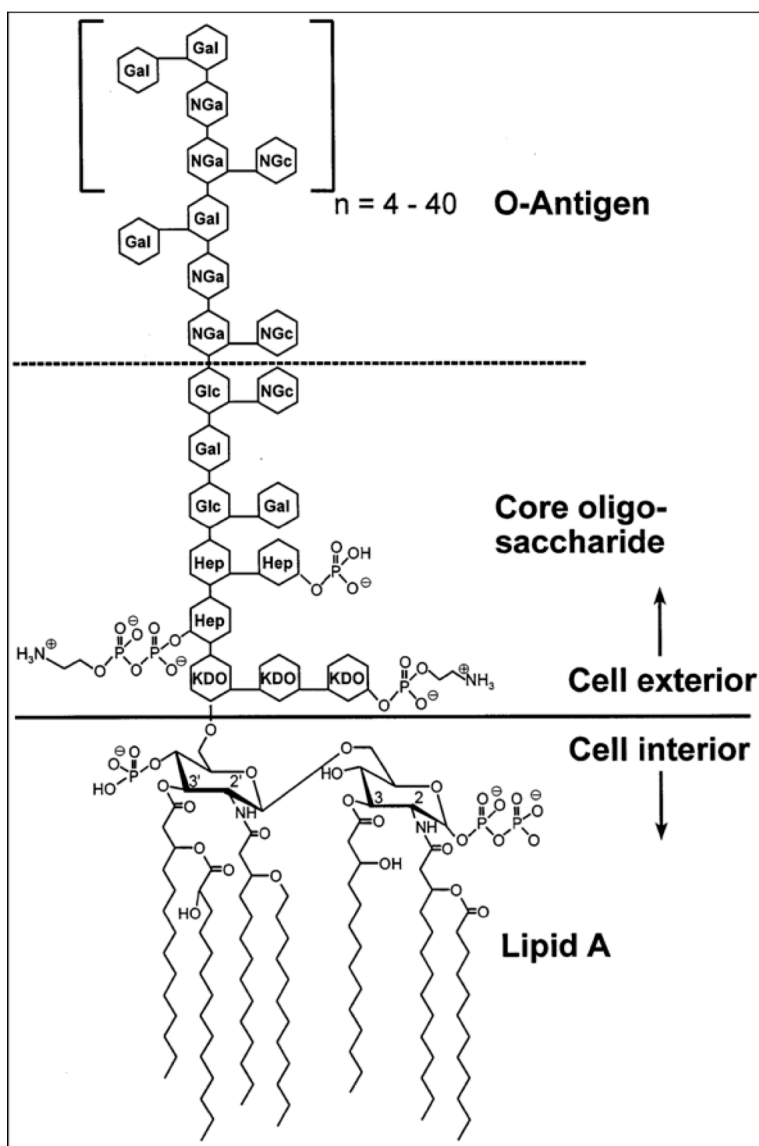


Figure 6. Diagram of the chemical structure of lipopolysaccharide from *E. coli* (Magalhães et al., 2007).

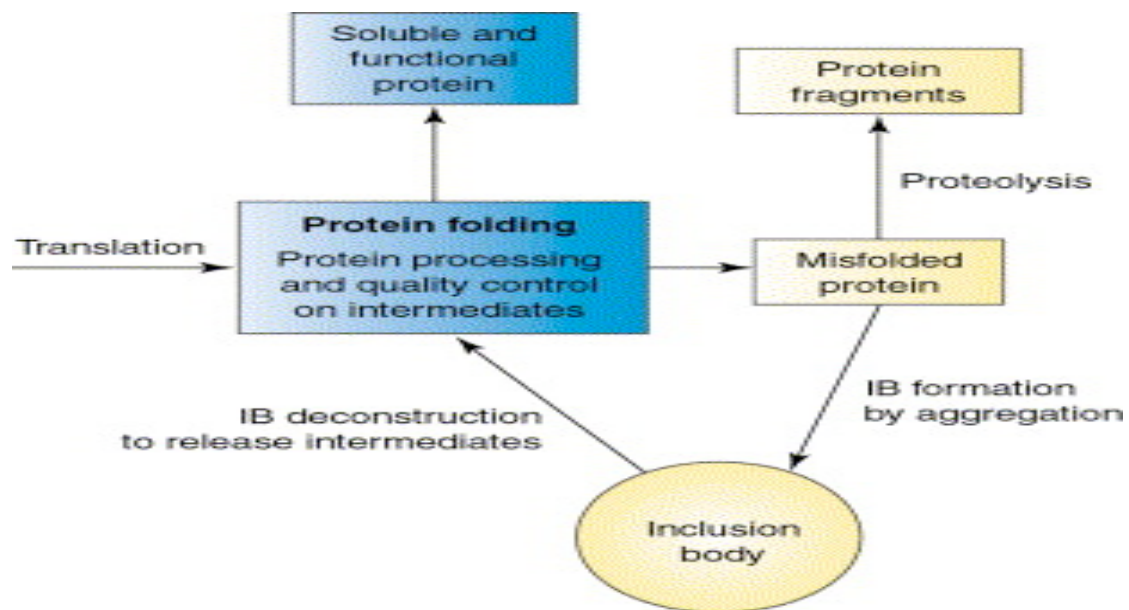


Figure 7. Schematic of soluble and insoluble protein formation when recombinant proteins are overexpressed in *E. coli*. (Middleberg, 2002).

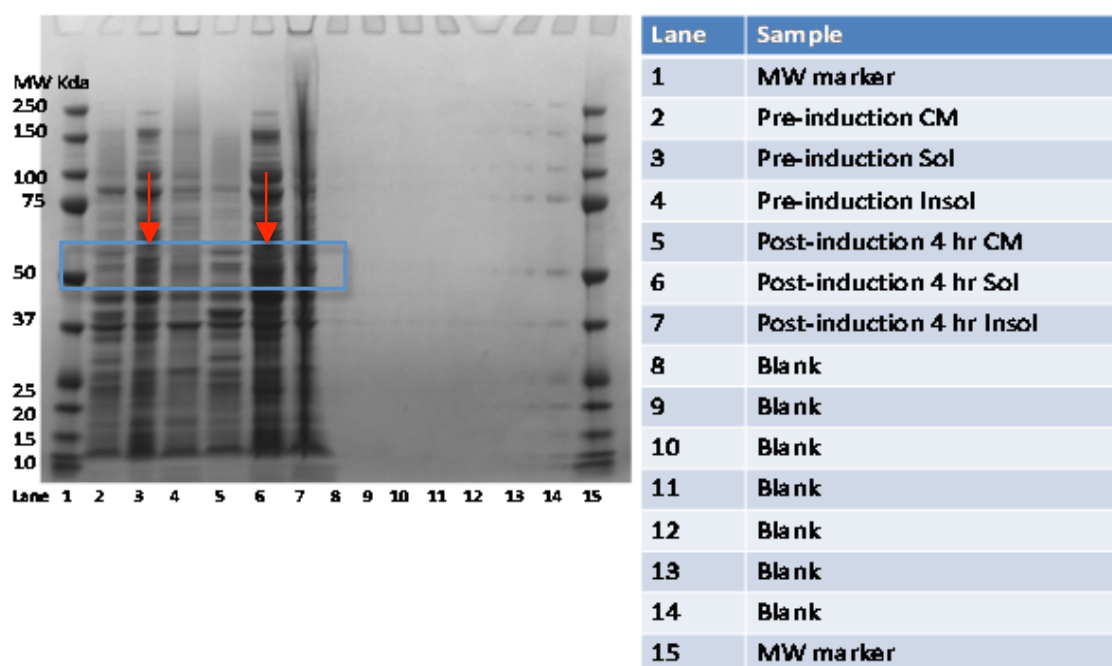


Figure 8. SDS-PAGE of soluble and insoluble protein fractions from fermentation samples before and after induction of recombinant FA4000 protein. The highlighted area indicates the location of the FA4000 protein and the arrows indicate the location of pre-induction soluble FA4000 (lane 3) and 4h post-induction soluble FA4000 (lane 6).

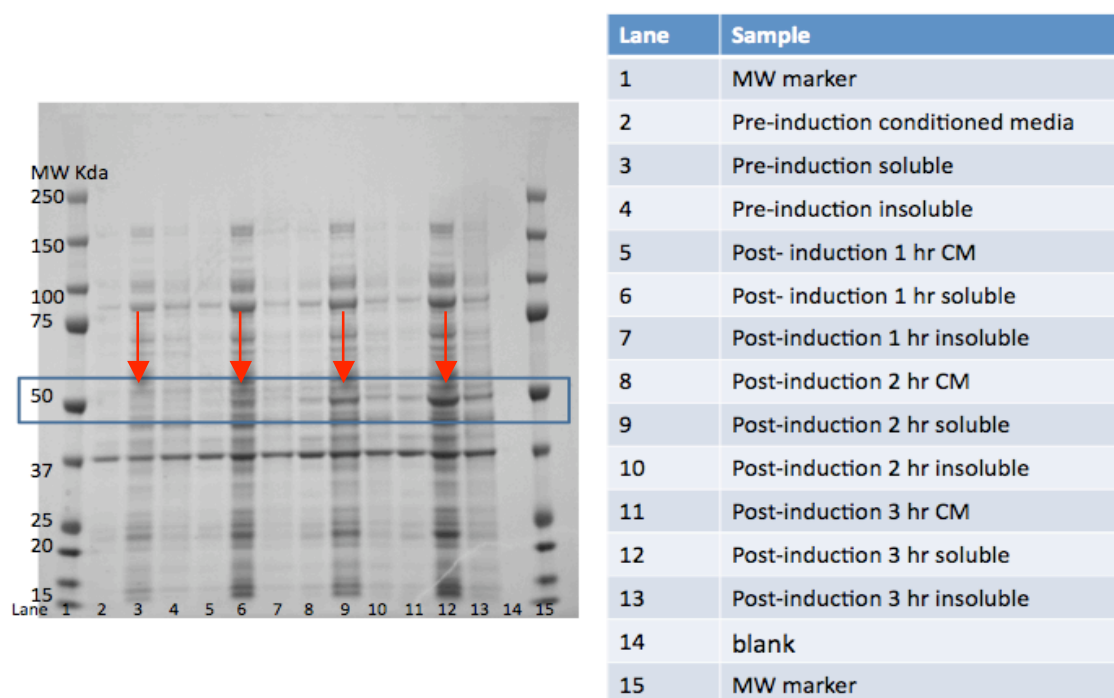


Figure 9. SDS-PAGE of soluble and insoluble protein fractions from fermentation samples before and after induction of recombinant 5800 protein. The highlighted area indicates the location of the recombinant 5088 protein and the arrows indicate the location of pre-induction soluble 5088 (lane 3), 1h post-induction soluble 5088 (lane 6), 2h post-induction soluble 5088 (lane 9) and 3h post-induction soluble 5088 (lane 12).

In the past, two purification schemes have been used at Georgia State University (Figure 10). The purification scheme used was dependent upon how the recombinant protein was expressed during fermentation. Both purifications began with homogenization to lyse the cells and an initial centrifugation step to remove cell debris. Following centrifugation, soluble protein present in the supernatant was purified using purification scheme A and insoluble protein present in the pellet was purified using purification scheme B. Because the recombinant FliC protein constructs in this study were expressed primarily as soluble protein only purification scheme A will be discussed. Purification scheme A was designed for protein expressed largely in the soluble fraction, but in order to optimize the protein yield it also incorporated steps to solubilize any insoluble protein still present in the supernatant. Each step in the purification was designed to remove one or more impurities using the physical and chemical properties of the recombinant protein and the methods chosen for purification are well-established techniques that can be easily and cost effectively scaled up for industrial scale production.

Polyethylene Glycol 3350 (PEG 3350) was chosen for protein precipitation. PEG 3350 is a parenthetical chemical widely used in chemical and biomedical applications and is non-denaturing, therefore maintaining the native structure of the protein and its biological activity. PEG 3350 also effectively precipitates protein at a low concentration, typically less than a 20% concentration of PEG 3350. The mechanism behind polyethylene glycol (PEG) precipitation is not well understood, but is thought to surround the protein causing it to precipitate from solution (Atha and Ingram, 1981). Application of PEG precipitation remains empirical, so each new recombinant FliC fusion protein will likely require optimization of PEG 3350 precipitation. Following the PEG precipitation, the

proteins were resuspended in a denaturing 8M urea buffer to recover the insoluble protein present in the cell lysate. While this allows the recovery of insoluble protein, it also denatures the already biologically active soluble protein present and necessitated the addition of several steps to the purification process: tangential flow filtration and a solubilization and refolding step.

A buffer exchange step using tangential flow filtration (TFF) was used to remove the denaturing agent present in the sample prior to the two-phase separation. TFF, or cross flow filtration, can be used to process large volumes of sample and involves the use of a membrane with a specific molecular weight cut-off. The sample was pumped against the membrane and filtration was achieved by creating pressure against the membrane pushing solute and smaller molecules through the membrane in the filtrate. The larger molecules, including the recombinant protein, that were not pushed through the membrane were collected in the retentate. During the filtration process, a non-denaturing buffer was continually added to the retentate in order to achieve a buffer exchange. Because the denaturing agent was removed during the buffer exchange, the protein refolded and must be denatured following the two-phase separation and then refolded again under controlled conditions to promote refolding into the proper biologically active conformation.

A two-phase separation using the surfactant, Triton® X-114, was used following the buffer exchange in order to remove endotoxin from the recombinant protein. Two-phase separation was chosen because it is a cost-effective method and has been shown successfully remove endotoxin from recombinant protein solutions (Aida and Pabst, 1990; Liu et al., 1997). Upon addition of Triton X®-114 at a temperature below 22°C, the cloud point for Triton X®-114, ET disassociates from the protein in a homogeneous solution.

When the temperature is raised above 22°C, the solution separates into 2-phases, an aqueous phase containing the recombinant protein and a micelle rich detergent phase containing the endotoxin (Figure 11). The aqueous and detergent phase were separated using centrifugation at a temperature above the cloud point.

The final steps in the purification included anion exchange chromatography (AEX) and hydrophobic interaction chromatography (HIC). AEX separates protein based on its net surface charge. Proteins in a solution with a pH higher than their isoelectric point (pI) have a negative charge, allowing the protein to bind reversibly to positively charged media in the AEX column. The bound protein is eluted by changing the ionic strength (salt concentration) of the buffer running through the column. This step was used to separate the recombinant protein from other proteins remaining as well as removing any residual Triton X®-114 or impurities still present. HIC was used as a polishing step when necessary to remove any remaining impurities. HIC separates proteins based on their surface hydrophobicity. The salt concentration of the solution containing the recombinant protein is increased prior to HIC to enhance the interaction of the hydrophobic components of the protein with the media in the HIC column. The proteins bind reversibly to the media in the column and are eluted from the column by decreasing the ionic strength (salt concentration) of the running buffer.

Once the recombinant FliC fusion protein was purified it was tested for TLR5 activity using modified Human Embryonic Kidney (HEK) 293 cells. Commercially available HEK-Blue™ hTLR5 cells (InvivoGen, San Diego, CA) (hTRL5 cells) were co-transfected with a human TLR5 (hTLR5) gene and secreted embryonic alkaline phosphatase (SEAP) gene under the control of a NF-κB promoter. Stimulation of hTLR5 by its ligand, flagellin,

activates the NF- κ B promoter inducing production of SEAP. The level of TLR5 activation was measured by detecting the amount of SEAP present due to the stimulation of hTLR5 through a color change. HEK-Blue™ Null I cells (InvivoGen, San Diego, CA) (null cells) are the parental cell line for the HEK-Blue™ hTLR5 cells and were used in the assay to determine the baseline level of TLR5 activity in the cells. The TLR5 assay determined if the fusion protein was capable of inducing an immune response via binding to TLR5. The recombinant protein must exhibit a strong TLR5 response if it is to be used as a vaccine. Low or no TLR5 activity might indicate that the recombinant protein does not have the proper conformation needed to induce a TLR5 response and the construct needs to be reevaluated.

1.1. Rationale and Hypothesis

GSU does not have a purification scheme designed to purify only the recombinant flagellin (FliC) proteins expressed as soluble protein. Purification of the soluble recombinant protein would hypothetically reduce the number of steps presently used in purification scheme A, as well as the time and cost needed for purification. By purifying the soluble protein the denaturing step following the PEG precipitation would be eliminated, which would also allow the removal of the buffer exchange via TFF, denaturing step following the two-phase separation and the refolding step. Currently, there is no standard protocol for refolding proteins and this process alone can account for a major portion of production costs (Datar et al., 1993). The protein would also remain in its native, biologically active form throughout the purification process and it is hypothesized that the soluble recombinant flagellin protein should have a higher TLR5 activity than recombinant protein that has been refolded. The recombinant proteins previously purified at GSU using either purification scheme A or B have exhibited TLR5 activity at a lower level than a commercially available recombinant FliC standard and the amount of recombinant protein necessary to elicit a comparable TLR5 response has been significantly higher than the FliC standard. A purification strategy designed for the soluble fraction of recombinant proteins FA4000 and 5088 could also potentially be applicable to future *Salmonella typhimurium* FliC vaccine fusion proteins.

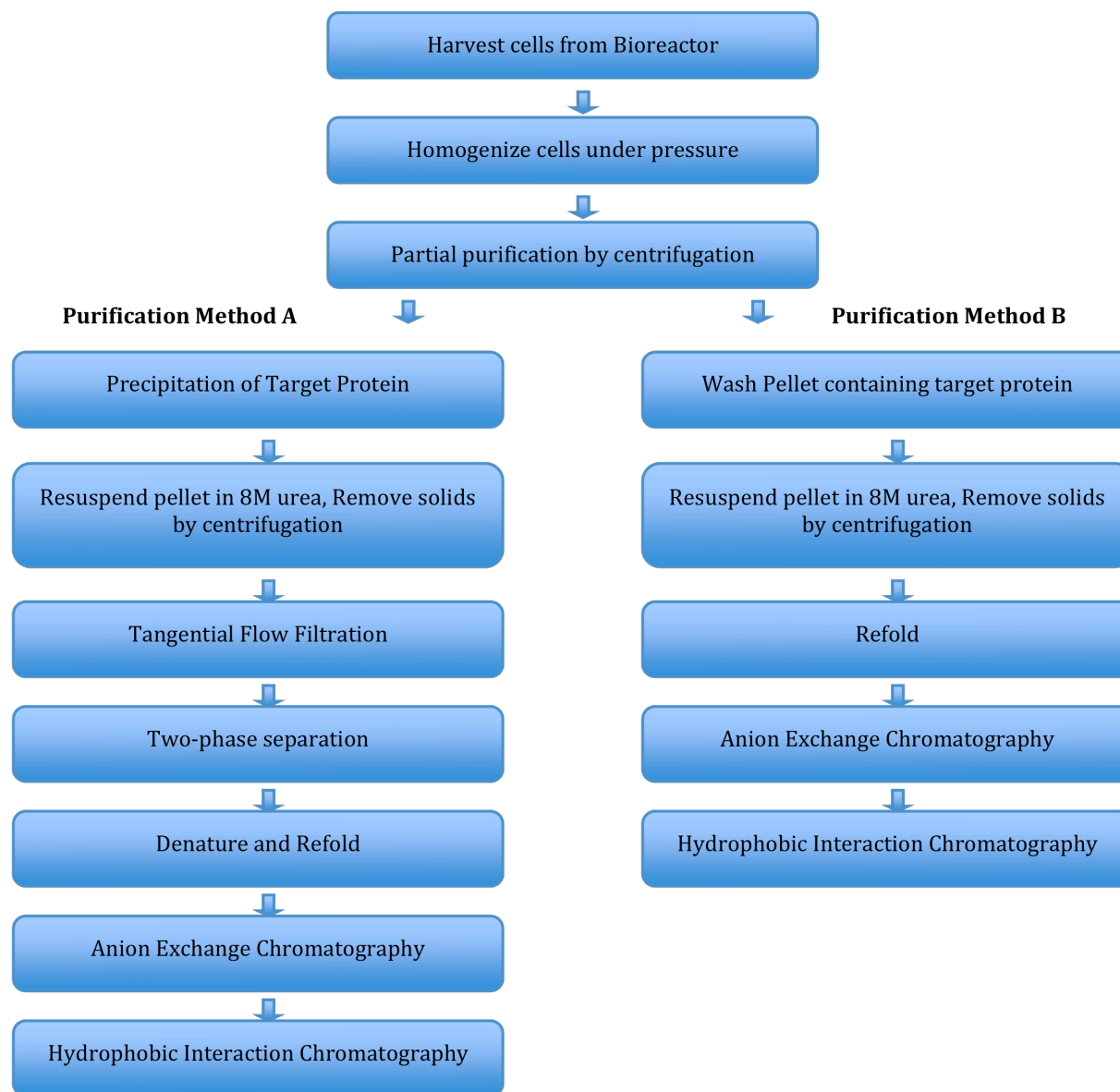


Figure 10. Schematic of Applied and Environmental Microbiology (GSU) Purification Processes for A) Purification process for both soluble and insoluble protein and B) Purification process for insoluble protein.

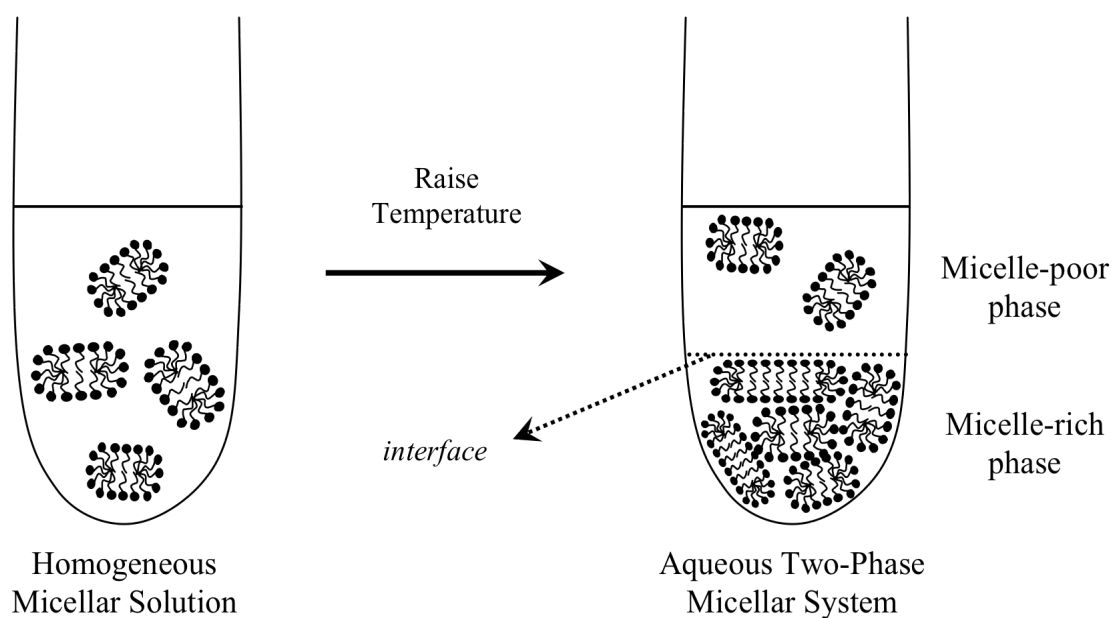


Figure 11. Illustration of formation of phase separation in Triton® detergents above their cloud point. For Triton® X-114, the two phases form above the cloud-point temperature of 22°C, allowing separation of the protein and endotoxin.

2. MATERIALS AND METHODS

2.1. Homogenization of Cell Paste

The same homogenization method was used for both FA400 and 5088 FliC recombinant proteins. The cells harvested from the fermentor was stored at -80°C in 150g aliquots. Prior to homogenization, the cell paste was removed from -80°C and allowed to thaw overnight at 4°C. The weight of the cell paste was used to calculate the amount of Lysis Buffer (20mM Tris, 100mM NaCl, 1mM EDTA, 100mM sucrose, pH 8) to add, with the volume of lysis buffer added equal to 15% solids. The thawed cell paste and lysis buffer were placed in an appropriate container with a stir bar and allowed to stir for > 30 minutes on ice. To prepare the APV®-1000 homogenizer (APV® Delavan, WI) for the sample, cold ddH₂O was run continuously through the homogenizer for 10 minutes, followed by cold lysis buffer for another 5-10 minutes. The resuspended cell paste was sampled and then poured into the hopper on the homogenizer and passed through the homogenizer at 0 bar into an appropriate sized container in an ice bath containing ice and isopropyl alcohol. The cell paste was then passed through the homogenizer 3 more times at 690bar. The temperature of the cell lysate was monitored after each pass through the homogenizer to ensure the temperature remained below 25°C and allowed to cool to 5°C before passing through the homogenizer again. After the final pass, the cell lysate was collected, the volume measured, sampled and either stored at 4°C or further processed.

2.2. Partial Clarification and Polyethylene Glycol 3350 Protein Precipitation

The cell lysate was centrifuged for 30 minutes at 4°C, 10,000rpm to remove cell debris. The supernatant was poured off, the volume measured and retained for further processing. The pellet was weighed, resuspended in an 8M urea buffer equal to the volume of the supernatant, sampled for analysis and discarded once testing was completed and showed no significant loss of the target protein. In order to precipitate the protein of interest, a 4% concentration of PEG 3350 was added to the supernatant collected from the centrifugation step above. The PEG 3350 was added slowly to the supernatant while stirring. After all the PEG 3350 dissolved, the supernatant was allowed to stir for ≥ 20 minutes. The solution containing the precipitated recombinant proteins was centrifuged for 1 hour at 4°C, 10,000rpm. (Following this step, the PEG 3350 pellets can be frozen at -20°C to be processed at a later date.) After centrifugation, the supernatant was pooled and sampled, the volume measured and then discarded. The pellet was resuspended in Resuspension Buffer (20mM tris, 1mM EDTA, 100mM sucrose, pH8) equal to $\frac{1}{2}$ the volume of the supernatant and stirred for ≥ 30 minutes until the pellet was completely resuspended. After sampling the resuspended PEG 3350 pellet, the pellet was centrifuged for 1 hour at 4°C, 10,000rpm. The resulting pellets were pooled, weighed, resuspended in an 8M urea buffer equal to the volume of the supernatant, sampled and then discarded. The supernatant or refined lysate (RL) was pooled and further processed using two-phase separation and hydrophobic interaction chromatography (HIC) or stored at 4°C.

2.3. Two-phase separation

1% (w/w) Triton® X-114 was added to the RL and stirred for ≥ 30 minutes at room temperature. The RL was centrifuged for 4 hours at 24°C, 10,000 rpm with the centrifuge brake off and left overnight. Following centrifugation the top layer or aqueous phase (AP) was carefully removed using a pipet, pooled, sampled and filtered using a 0.2 μ m polyethersulfone (PES) syringe filter and either stored at 4°C or further processed. The detergent phase (DP) was pooled, sampled and discarded.

2.4. Hydrophobic Interaction Chromatography

The filtered aqueous phase (FAP) was diluted to 2.5M NaCl with a 1:1 dilution of 5M sodium chloride (NaCl) to FAP and stirred for 10 minutes. Prior to loading the FAP on a 5ml Toyopearl® PPG-600 HIC column (Tosoh Bioscience, King of Prussia, PA), the sample was filtered using a 0.2 μ m PES syringe filter and the protein was diluted to 0.1mg/ml total protein with 2.5M NaCl. Column chromatography was performed using the ÄKTAexplorer™ 100 system. (GE Lifesciences, Piscataway, NJ) The column was equilibrated for 5 column volumes (CV) with equilibration buffer (2.5M NaCl, 20mM tris, pH 8). Following equilibration 10 milligrams of diluted FAP was loaded onto the column using the sample pump, P-960 and washed with 2CV of equilibration buffer (2.5M NaCl, 20mM tris, pH 8) to remove any unbound protein. During the previous two steps, the load flow through and post load wash were collected separately for testing. Bound protein was eluted by decreasing the salt concentration using a step elution of 25%, 50%, 75% and

100% 20mM tris, pH8. The eluate was collected using a Fraction Collector Frac-950 (GE Lifesciences, Piscataway, NJ) in 5ml fractions and the fractions corresponding to each peak were collected for further processing and testing.

2.5. Sample Desalting

Following HIC, the samples were desalted using a HiPrep™ 26/10 desalting column and ÄKTAexplorer™ 100 system (GE Lifesciences, Piscataway, NJ) or a 10ml Dextran desalting column (Thermo Scientific, Rockford, IL) depending on sample size. The columns were equilibrated with 5 CV of 20mM tris, pH 8. Following the column equilibration, the sample was loaded via sample pump, P-960 when using the HiPrep™ 26/10 desalting column or via pipet for the 10ml dextran desalting column. The protein was eluted with 1-2 CV 20mM tris and the eluate was collected using a Fraction Collector Frac-950 (GE Lifesciences, Piscataway, NJ) in 5ml fractions for the HiPrep™ 26/10 desalting column. Eluate from the 10ml dextran desalting column were collected manually in 0.5ml fractions.

2.6. SDS-PAGE Gel Electrophoresis

Running buffer for gel electrophoresis was prepared by adding 50ml of 20X NuPAGE® MOPS Running Buffer (Invitrogen, Grand Island, NY) to 950ml ddH₂O for a 1X MOPS Running Buffer. (Running buffer should be prepared the day prior to running the gel, so the running buffer can be chilled to 4°C. This helps to dissipate the heat generated when the gel is being run.) The samples to be imaged were prepared for SDS-PAGE electrophoresis by adding 5µg of sample protein to 10µl NuPAGE® LDS Sample Buffer (Invitrogen™, Grand Island, NY) and ddH₂O up to 40µl in a 1.5ml micro-centrifuge tube.

For samples with a protein concentration lower than 0.6mg/ml, 30µl of sample and 10µl NuPAGE® LDS Sample Buffer (Invitrogen™, Grand Island, NY) was used. The samples were incubated for 10 minutes at 70°C and allowed to cool. Prior to loading the samples 1X MOPS running buffer was added to the upper chamber so that the wells in the gel are filled with buffer and to determine that there was no buffer leaking from the upper buffer chamber. 10µl of each sample was loaded into a 15 well NuPAGE® Novex 4-12% Bis-Tris Gel (Invitrogen, Grand Island, NY) and 5µl of MWM added to at least one well. Once the samples were loaded 1X MOPS running buffer (~600ml) was added to the outer buffer chamber. The SDS-PAGE gels were run in the Xcell Surelock® Mini-Cell (Invitrogen, Grand Island, NY) at 200V for 1 hour.

2.7. Coomassie Blue Staining

A stain of 1% Coomassie® Brilliant Blue G-250 (Sigma) was prepared and filtered prior to use. The SDS-PAGE gels containing sample were stained for a minimum of 3 hours to overnight. Following staining, the gels were destained using ddH₂O until the protein bands were visible and the gel able to be imaged.

2.8. Kinetic –QCL Endotoxin Assay

The endotoxin level of each sample was determined using the Lonza QCL-kinetic chromogenic kit® (Lonza, Allendale, NJ), which detects Gram-negative bacterial endotoxin. The samples were pre-diluted using limulus amebocyte lysate (LAL) reagent water to a lower concentration in order to fit into the range of the standard curve. The dilution was dependent on which stage of purification the sample was collected. The standard for the assay was an *E. coli* control standard endotoxin (CSE) that was rehydrated with a pre-determined amount of LAL reagent water indicated on the certificate of analysis to yield a concentration of 50EU/ml. Prior to diluting the CSE, it was incubated at room temperature for 15 minutes and then vortexed for 15 minutes. The CSE was diluted using LAL reagent water to several concentrations in order to generate a standard curve. (Table 2) The samples were also diluted to several concentrations in order to fit within the standard curve and determine the endotoxin concentration of each sample (Table 3).

Table 2. *E. coli* control standard endotoxin dilutions for LAL endotoxin assay

Dilution	Sample Volume(μL)	Diluent (μL)	Total Volume(μL)
1:5	80	320	400
1:25	80 of 1:5	320	400
1:125	80 of 1:25	320	400
1:625	80 of 1:125	320	400

Table 3. Sample dilutions for LAL endotoxin assay

Dilution	CSE Volume (μL)	Diluent (μL)	Total Volume(μL)
1:5	80	320	400
1:50	50 of 1:5	450	500
1:500	50 of 1:50	450	500
1:5000	50 of 1: 500	450	500

Each CSE and sample dilution was vortexed for ~ 1 minute between each serial dilution and vortexed again before it was added to a 96-well microplate. Following addition of the samples, the microplate was pre-incubated for 10 minutes at 37°C prior to adding the LAL reagent. Before the 10-minute pre-incubation was over the LAL reagent was rehydrated with 2.6ml LAL reagent water and shaken until fully dissolved. The rehydrated LAL reagent was added to a pyrogen-free reservoir. Following the 10 minute incubation of the microplate, 100 µL of the LAL reagent was added to each well containing sample using a multichannel pipette. (It is important that there are no bubbles are present in the plate wells.) The plate was loaded into the SpectraMax® Plus Microplate Reader (Molecular Devices, Sunnyvale, CA) and absorbance (405nm) was read every 2½ minutes at 37°C until the absorbance increased 0.200 absorbance units from the first reading. The data was collected and analyzed using SoftMax® Pro software (Molecular Devices, Sunnyvale, CA) and a standard curve was generated with a log-log curve. The correlation coefficient (r) should be ≥ 0.980 in order to construct the standard curve and predict the sample endotoxin concentration (x). The x value for each sample is then multiplied by their respective dilution factor to obtain the total amount of endotoxin for each sample.

2.9. Maintenance of HEK-Blue™ hTLR5 and HEK-Blue™ Null I cells

2.9.1. Reviving HEK-Blue™ hTLR5 and HEK-Blue™ Null I cells

Both HEK-Blue™ hTLR5 (hTLR5) and HEK-Blue™ Null I (null) cells were stored in 1ml aliquots in cryovials in liquid nitrogen prior to use. To start a new culture for testing, a vial of each type of cell was removed from liquid nitrogen and placed in a 37°C water bath to thaw. Two T75 flasks (Corning, Tewksbury, MA) for each type of HEK cell were filled with 11.5ml of growth media (DMEM w/ 4.5 g/l glucose, 10% (v/v) fetal bovine serum, 50 U/ml penicillin, 50 µg/ml streptomycin, 100 µg/ml Normocin™, 2mM L-glutamine) previously warmed to 37°C in a water bath. 0.5ml of thawed cells were added to the growth media in the flasks and gently swirled. The flasks were placed in a 37°C, 5% CO₂ incubator. The cells were allowed to grow until they were 70-80% confluent before passaging the cells to new T75 flasks. The cells were passaged twice after removal from storage before the growth media was supplemented with Blasticidin (30 µg/ml) and Zeocin™ (100 µg/ml) for hTLR5 cells and Zeocin™ (100 µg/ml) for the null cells. The growth media was changed twice a week.

2.9.2. Passaging HEK-Blue™ hTLR5 and HEK-Blue™ Null I cells

Cells were passaged when growth was 70-80% confluent. The spent media was removed and the cells were washed with 5ml 1X phosphate buffered saline (PBS) (Corning, Tewksbury, MA). The PBS was removed using a pipet and another 5ml 1X PBS added to the flask to resuspend the cells. The cells were removed from the flask wall by gently tapping the flask. Once the cells had been removed from the flask wall, the cells were pipetted up and down to separate the cells from each other. Two T75 flasks (Corning, Tewksbury, MA)

for each cell were filled with 11.5ml of growth media (hTLR5 - DMEM w/ 4.5 g/l glucose, 10% (v/v) fetal bovine serum, 50 U/ml penicillin, 50 µg/ml streptomycin, 100 µg/ml Normocin™, 2mM L-glutamine, 30 µg/ml Blasticidin, 100 µg/ml Zeocin™ and null - DMEM w/ 4.5 g/l glucose, 10% (v/v) fetal bovine serum, 50 U/ml penicillin, 50 µg/ml streptomycin, 100 µg/ml Normocin™, 2mM L-glutamine, 100 µg/ml Zeocin™) warmed to 37°C. 0.5ml of resuspended cells were added to the growth media in the flasks and gently swirled. The flasks were placed in a 37°C, 5% CO₂ incubator.

2.10. TLR5 Activity Assay

Using HEK-Blue™ hTLR5 cells (InvivoGen, San Diego, CA) (hTRL5 cells) stimulation of TLR5 by its ligand, flagellin, was determined by measuring changes in color. HEK-Blue hTLR5 cells were co-transfected with a human TLR5 (hTLR5) gene and secreted embryonic alkaline phosphatase (SEAP) gene under the control of a NF-κB promoter. Stimulation of hTLR5 activates the NF-κB promoter inducing production of SEAP. QUANTI-Blue™ (InvivoGen, San Diego, CA) was used to detect the amount of alkaline phosphatase present due to the stimulation of hTLR5. Color changes were measured by OD₆₃₀. Recombinant *Salmonella typhimurium* flagellin or FliC (InvivoGen, San Diego, CA) was used as the positive control and endotoxin free water as a negative control. HEK-Blue™ Null I cells (InvivoGen, San Diego, CA) (null cells) are the parental cell line for the HEK-Blue™ hTLR5 cells and were used in the assay to determine the baseline level of TLR5 activity in the cells.

Both the hTLR5 and null cells were harvested from T75 tissue culture flasks (Corning®, Tewksbury, MA) with 5-10ml 1x PBS (Corning cellgro®, Tewksbury, MA).

The cells were diluted with a 0.4% trypan blue solution (Sigma-Aldrich®, St. Louis, MO) and counted using a Neubauer hemocytometer. Once the total number of cells/ml was calculated, the cells were diluted with test media (DMEM, 4.5g/l glucose, 50U/ml penicillin, 50µg/ml streptomycin, 100µg/ml Normocin™, 2mM L-glutamine, 10% (v/v) heat-inactivated fetal bovine serum) to a concentration of 140,000 cells/ml. 20µl of each sample in triplicate, as well as the positive control and negative control were added to a 96 well plate. 180µl of the above cell suspension was added to each well with sample. The plate was incubated at 37°C, 5% CO₂ for 20-24 hours. Following incubation, 20µl of supernatant from each sample was added to a new well and 180µl of QUANTI-Blue™ was added to the supernatant. The plate was incubated for 1-3 hours at 37°C. After incubation the absorbance of each well was read at 630nm with a Victor™ Plate Reader (PerkinElmer, Waltham, MA). To determine the level of TLR5 activity, the OD₆₃₀ sample reading of the HEK-Blue™ Null I cells was subtracted from the OD₆₃₀ reading of the HEK-Blue™ hTLR5 cells to determine the accurate TLR5 activity for each sample.

2.11. Western Blot

The samples for Western Blotting were run on an SDS-PAGE gel with the Magic Mark™ XP Western Protein Standard (Invitrogen, Grand Island, NY) using the same protocol for SDS-PAGE gel electrophoresis. The gel was placed in water after it had finished running. The blotting pads were soaked in 700ml of transfer buffer until saturated and then pressed until all the air bubbles were removed. A pre-cut nitrocellulose blotting membrane, 0.45µm pore size and filters (Invitrogen, Grand Island, NY) were briefly soaked in transfer buffer prior to putting together the blotting “sandwich” (Figure 11). The

transfer was performed using the Xcell II™ Blot Module (Invitrogen, Grand Island, NY) at 360mA for 1hour. Upon completion of the transfer the blotting membrane was placed in a blocking solution 0.5% milk solution and incubated for 1 hour to overnight at 4°C in order to prevent non-specific binding of primary and/or secondary antibodies to the membrane. (When removing the blotting membrane from the “blotting sandwich”, it is important to pay attention to the orientation of the membrane or mark a corner so that the proteins can be correctly imaged and identified.) After incubation in the blocking solution the transfer membrane was washed 3 times for 10 minutes each in 50mM tris-Buffered Saline with 1% Tween20 (TBST). After washing, the membrane was incubated in 50ml TBST and 50µl of primary antibody, Anti-Flagellin (FliC) (Invivogen, San Diego, CA) for 1 hour, shaking at room temperature. Following incubation with the primary antibody, the membrane was washed 3 times for 10 minutes each in 50ml TBST. The membrane was then incubated in 50ml TBST and the secondary antibody, Rat anti-mouse IgG horseradish peroxidase (HRP) (Southern Biotech, Birmingham, AL) for 1 hour, shaking at room temperature. After incubation the membrane was again washed 3 times in 50ml TBST for 10 minutes each. The membrane was imaged using Western Lightning® Plus-ECL, Enhanced Chemiluminescence Substrate (Perkin Elmer, Waltham, MA) and the ImageQuant™ LAS 4000 (GE Lifesciences, Piscataway, NJ).

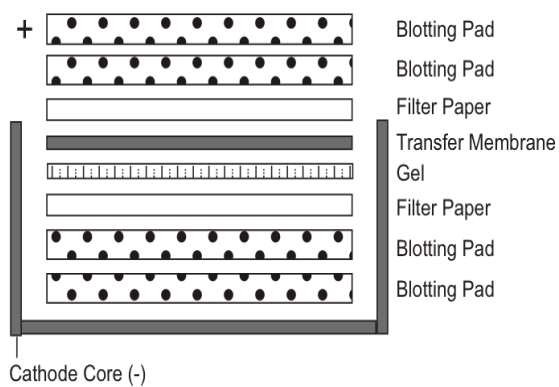


Figure 12. Set up for Western Blotting using Xcell II™ Blot Module.

3. RESULTS

3.1. Optimization of polyethylene glycol 3350 precipitation of soluble recombinant FA4000 and 5088 proteins

Several different concentrations of polyethylene glycol 3350 (PEG 3350) were evaluated to determine the most efficient concentration to precipitate FA4000 and 5088 recombinant proteins following homogenization and initial centrifugation. FA4000 protein was precipitated with a 4%, 6%, 9%, and 12% concentration of PEG 3350 and the 5088 protein with a 4%, 6%, 9%, 12%, and 15% concentration to determine which concentration precipitated the greatest amount of FA4000 and 5088 protein, in addition to removing a significant amount of the contaminating protein. Using SDS-PAGE and Coomassie® Blue staining to visually evaluate the results, a 4% (w/v) concentration of PEG 3350 yielded the best results for precipitation of both FA4000 and 5088. The recombinant proteins can clearly be seen in both the resuspended PEG 3350 pellet and refined lysate (RL) for FA4000 (Figure 12) and in the RL for 5088 (Figure 13). For 5088, a second band just below the recombinant protein was precipitated out at concentrations higher than 6% PEG 3350 making it appear that more recombinant protein had been precipitated. There was some loss of FA4000 and 5088 in the PEG 3350 supernatant, but the amount was not significant. Concentrations greater than 4% PEG 3350 did not increase the amount of recombinant protein present in either the resuspended PEG 3350 pellet or the RL for either FA4000 or 5088. A lower concentration of 2% PEG 3350 was also evaluated, but the pellet was too small for efficient processing.

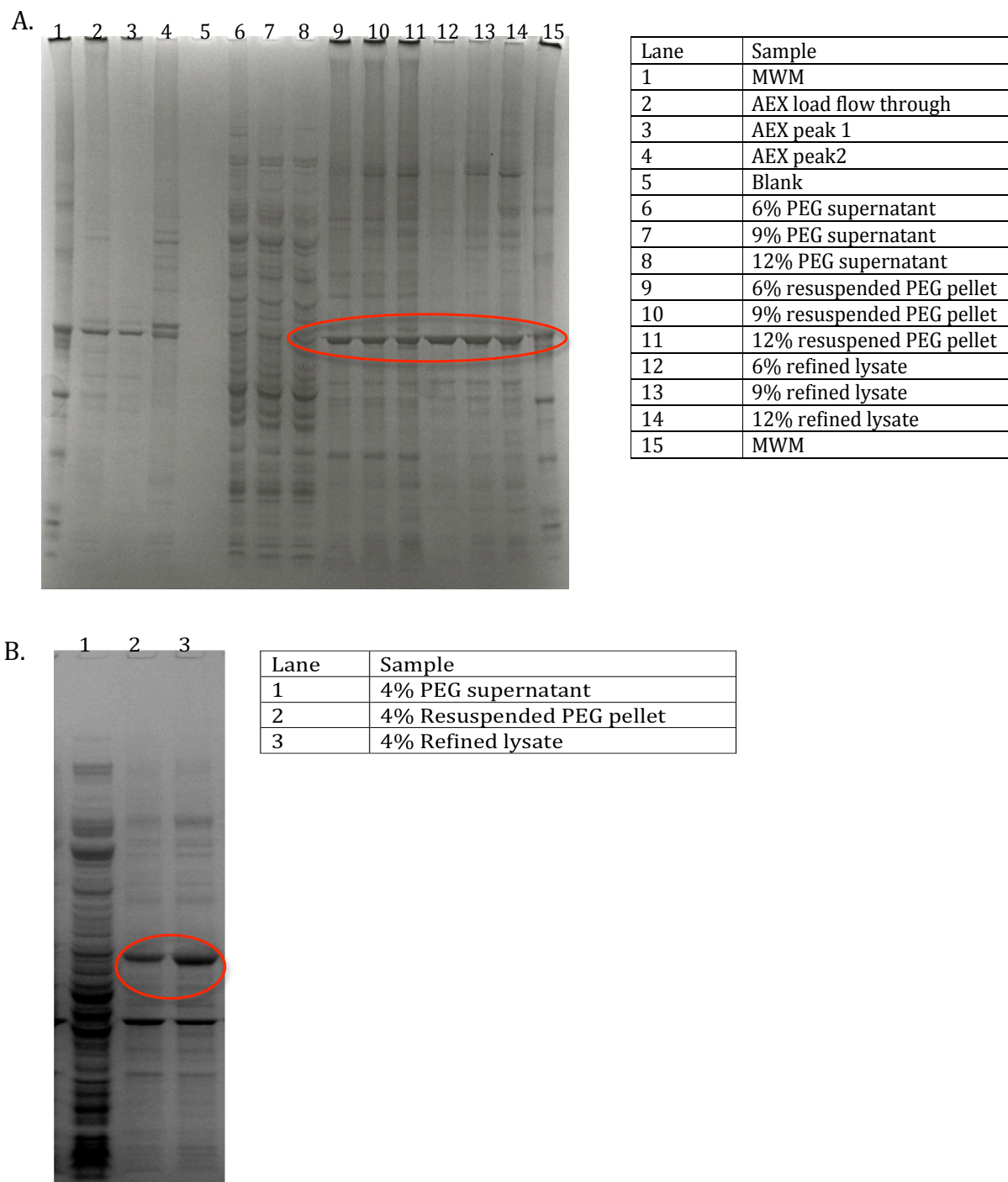


Figure 13. SDS-PAGE of FA4000 protein precipitated using different concentrations of PEG 3350 during purification. The highlighted areas indicate the location of the putative FA4000 protein. A. 6%, 9% and 12% PEG 3350 precipitation. Lanes 9-11 show putative FA4000 protein present in 6%, 9% and 12% resuspended PEG pellet. Lanes 12-14 show putative FA4000 protein present in 6%, 9%, and 12% refined lysate. B. 4% PEG 3350 precipitation. Lanes 2 & 3 show putative FA4000 protein present in 4% resuspended PEG pellet and refined lysate, respectively.

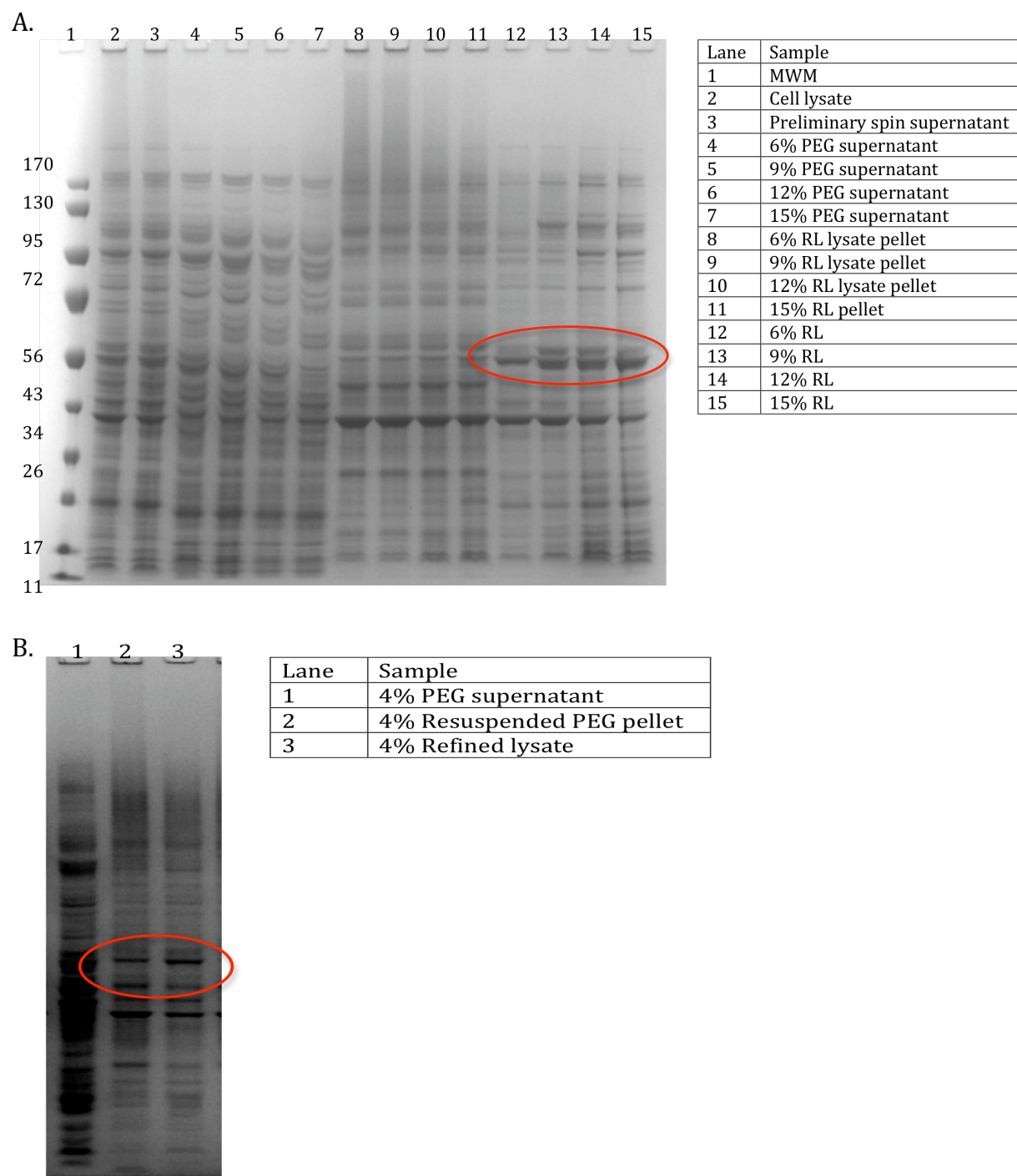


Figure 14. SDS-PAGE of 5088 protein precipitated using different concentrations of PEG 3350 during purification. The highlighted areas indicate the location of the putative 5088 protein. A. 6%, 9%, 12% and 15% PEG 3350 precipitation. Lanes 12-14 show presence of putative 5088 protein in refined lysate. B. 4% PEG 3350 precipitation. Lanes 2 & 3 show presence of putative 5088 protein in 4% resuspended PEG pellet and refined lysate, respectively.

3.2. Purification of soluble FA4000 and 5088 recombinant proteins

Each step of the purification process was designed to remove a specific or several impurities in order to obtain an acceptable level of purity in the protein. Samples were taken at designated steps in the process to determine the presence of the target protein using SDS-PAGE and protein purity (Appendix B) and the results determined whether to continue to the next purification step. Following homogenization, initial centrifugation and precipitation of the recombinant proteins using a concentration of 4% PEG 3350, the putative FA4000 and 5088 proteins can be seen in the refined lysate (RL), aqueous phase (AP) and filtered aqueous phase (FAP) samples run on SDS-PAGE and stained with Coomassie® Blue (Figures 14 & 15). There was also a significant reduction of contaminating proteins and increase in the relative amount of FA4000 and 5088 protein present in the samples. Following the 2-phase separation, the endotoxin level of the AP was reduced from 10^7 EU/ml in the cell lysate to 10^3 EU/ml for both FA4000 and 5088. Some loss of FA4000 and 5088 proteins was seen in the PEG 3350 supernatant and RL pellet, but this was expected during processing especially in centrifugation steps.

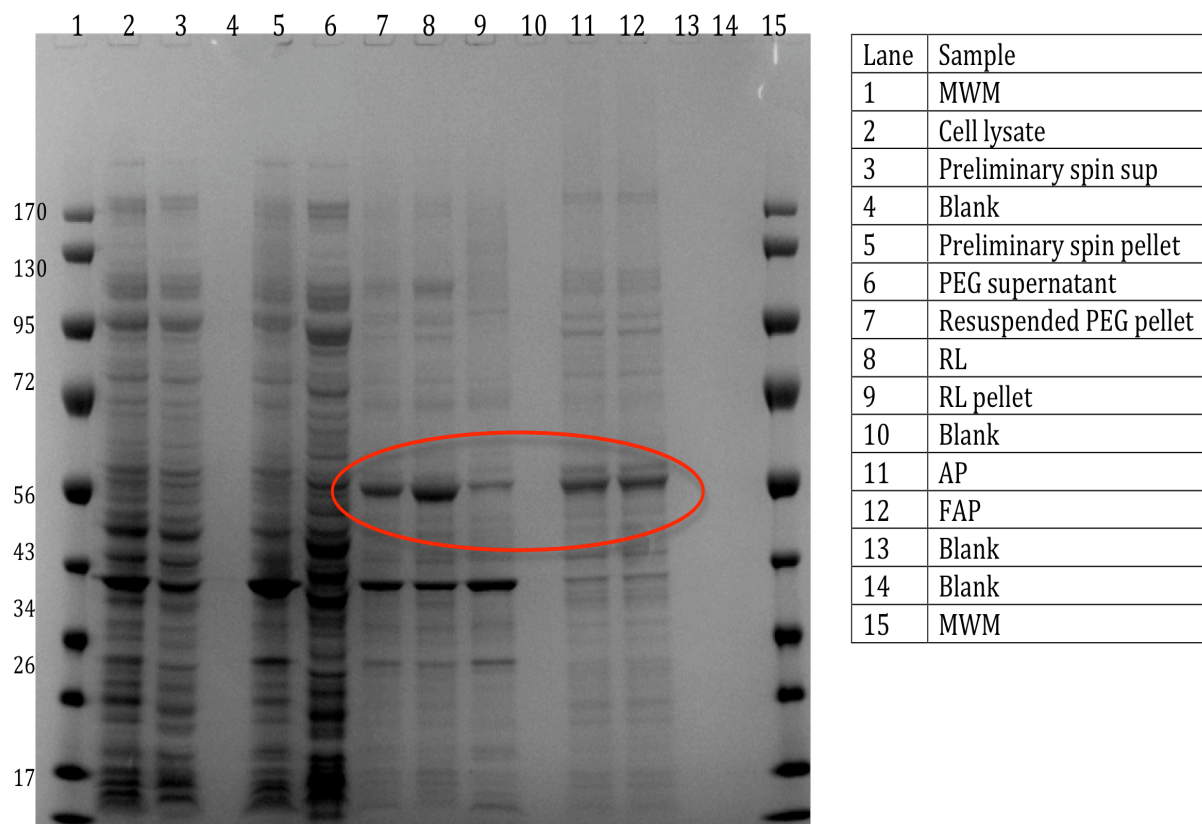


Figure 15. SDS-PAGE of putative FA4000 protein purification samples taken through two-phase separation step. The highlighted area indicates the location of putative FA4000 seen in the resuspended PEG 3350 pellet (lane 7), RL (lane 8) AP (lane 11) and FAP (lane 12).

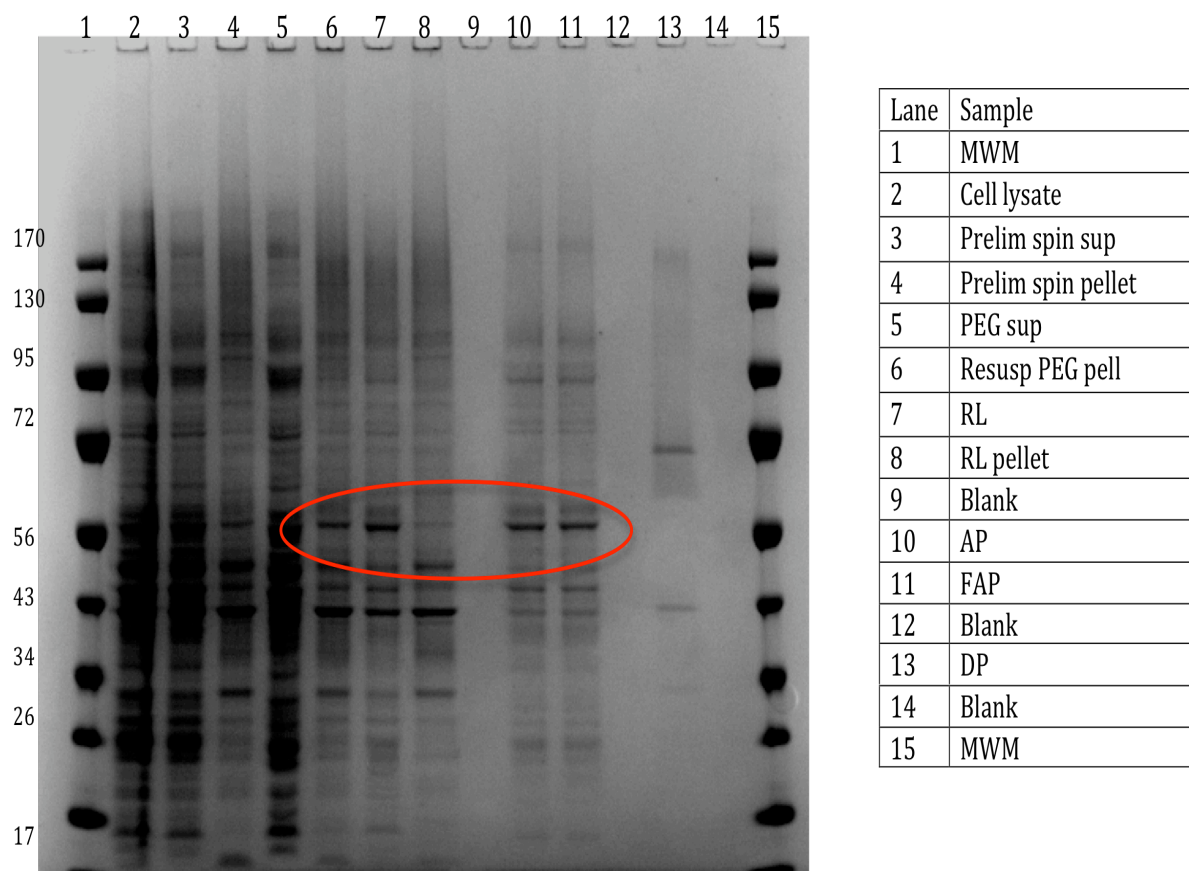


Figure 16. SDS-PAGE of putative 5088 protein purification samples taken through 2-phase separation step. The highlighted area indicates the location of putative 5088 protein seen in the resuspended PEG 3350 pellet (lane 6), RL (lane 7), AP (lane 10) and FAP (lane 11).

Following the 2-phase separation, the FAP was loaded onto a Toyopearl® PPG-600 HIC column (Tosoh Bioscience, King of Prussia, PA) and decreasing the salt concentration of the buffer running through the column eluted the bound protein. The fractions corresponding to each protein peak indicated by a peak in the UV₂₈₀ were collected, run on a SDS-PAGE gel and stained with Coomassie® blue. SDS-PAGE results showed the putative FA4000 protein (approximately 55kDa) in fractions A8 and A9 corresponding to peak 2 on the HIC chromatogram (Figures 16 and 17). Fractions A8 and A9 were chosen for further evaluation and validation due to their purity seen in SDS-PAGE. The putative 5088 protein (approximately 52kDa) was present in in fractions A1 and A2 corresponding to peak 1 on the HIC chromatogram (Figure 18 & 19). These two fractions were selected based on their purity seen in SDS-PAGE for further processing and validation of recombinant 5088 protein. The recombinant protein yield for both FA4000 and 5088 from HIC was 12-15%. The endotoxin levels for FA4000 and 5088 following HIC were reduced to 10¹-10² EU/ml and 10² EU/ml, respectively from 10³ EU/ml in the FAP.

The final yield of purified FA4000 was 450mg-680mg protein and 380mg-600mg protein for 5088 from a 30L fermentation. The endotoxin levels were significantly reduced during the purification process, with the greatest decreases in endotoxin seen in the 2-phase separation step and HIC separation (Table 4) bringing ET to a 150EU/mg protein for FA4000 and 175EU/mg protein for 5088, a level acceptable for use as a vaccine. Protein concentrations for samples taken throughout the purification process are shown in Table 5.

Western Blot was used to confirm the presence of recombinant FliC in the FA4000 and 5088 proteins selected following HIC. Using a commercially available anti-flagellin

FliC, the Western Blot showed the presence of FliC in FA4000 fractions, A8 and A9, as well as fraction A1 for 5088 (Figure 20). FA4000 and 5088 were also identified as flagellin from *Salmonella enterica* and *Salmonella typhimurium*, respectively, by mass spectrophotometry (MALDI TOF/TOF) (Appendix C). *Salmonella typhimurium* is a subspecies of *S. enterica*.

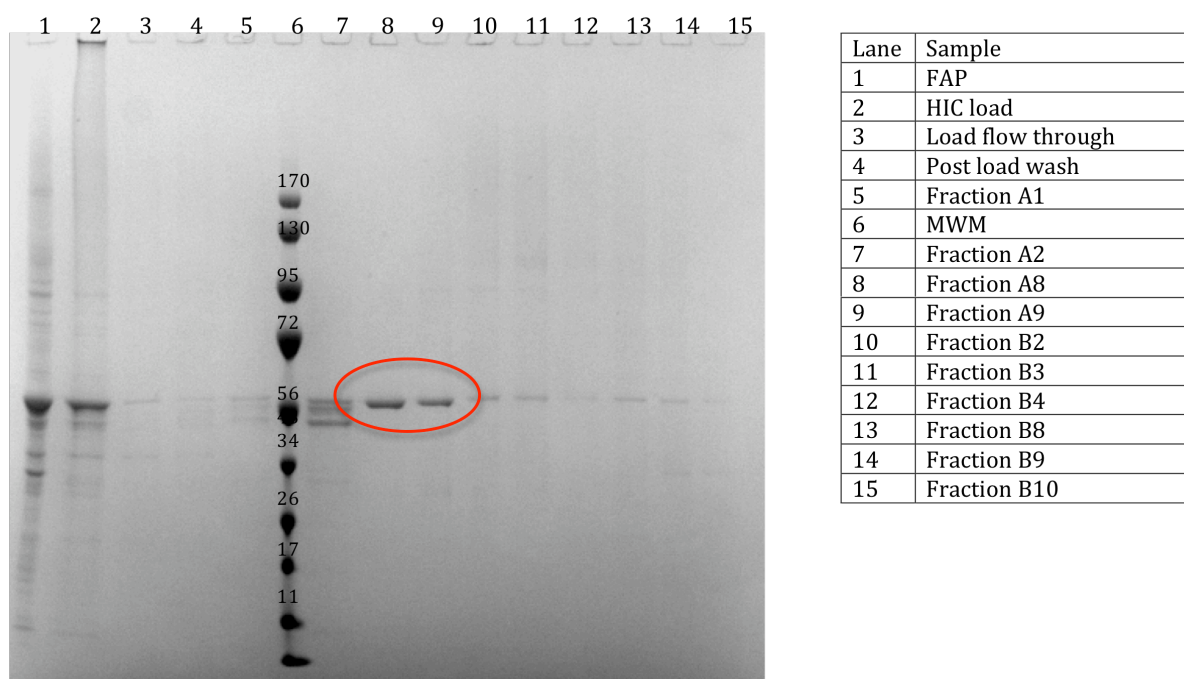


Figure 17. SDS-PAGE of hydrophobic interaction chromatography fractions for recombinant FA4000 protein. The highlighted area indicates the location of putative purified FA4000 protein in fractions A8 and A9 (lanes 8 and 9, respectively).

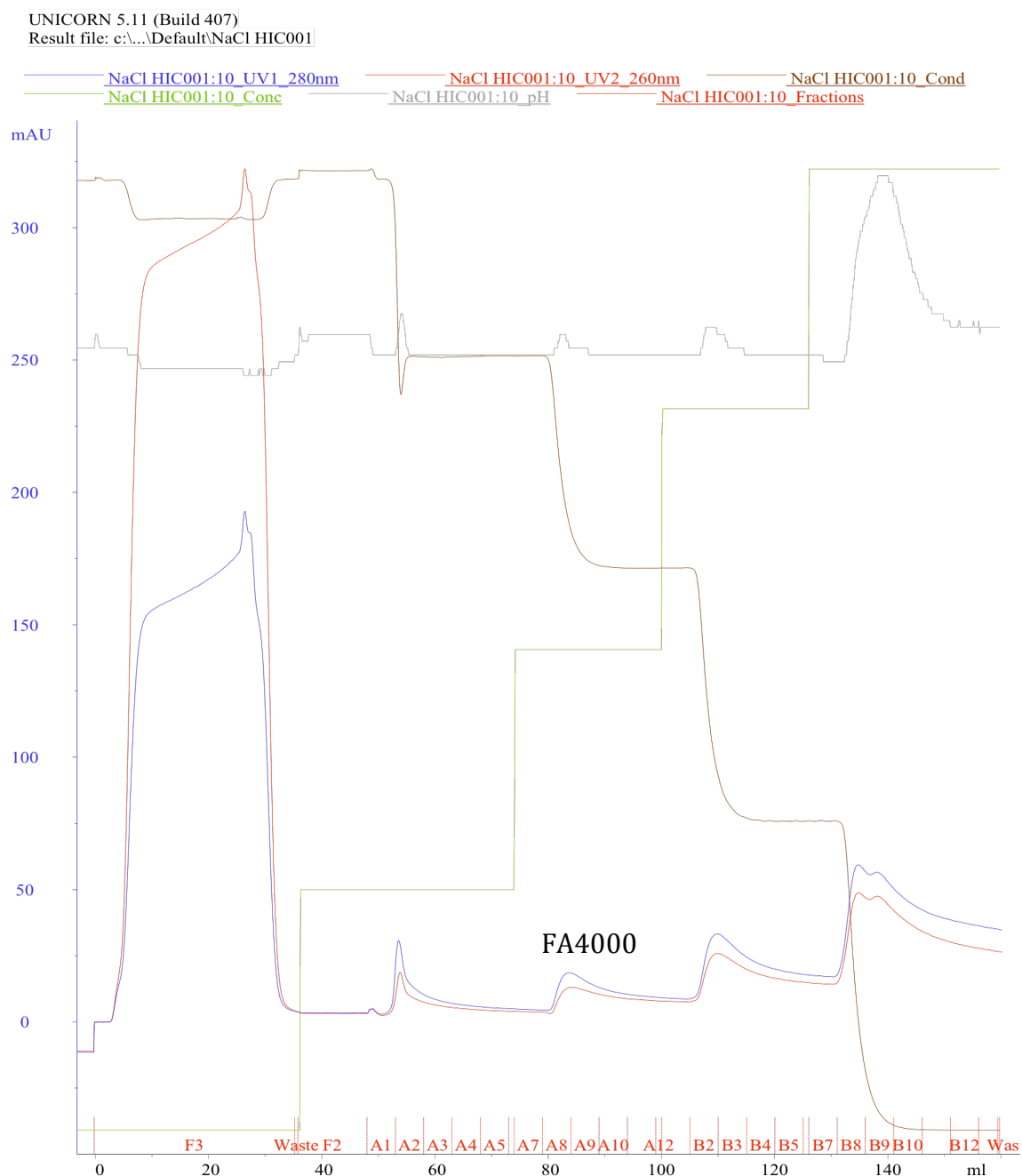


Figure 18. Chromatogram for hydrophobic interaction chromatography of recombinant FA4000 protein. Putative purified FA4000 protein was present in the second UV₂₈₀ peak following the loading step (fractions A8 and A9).

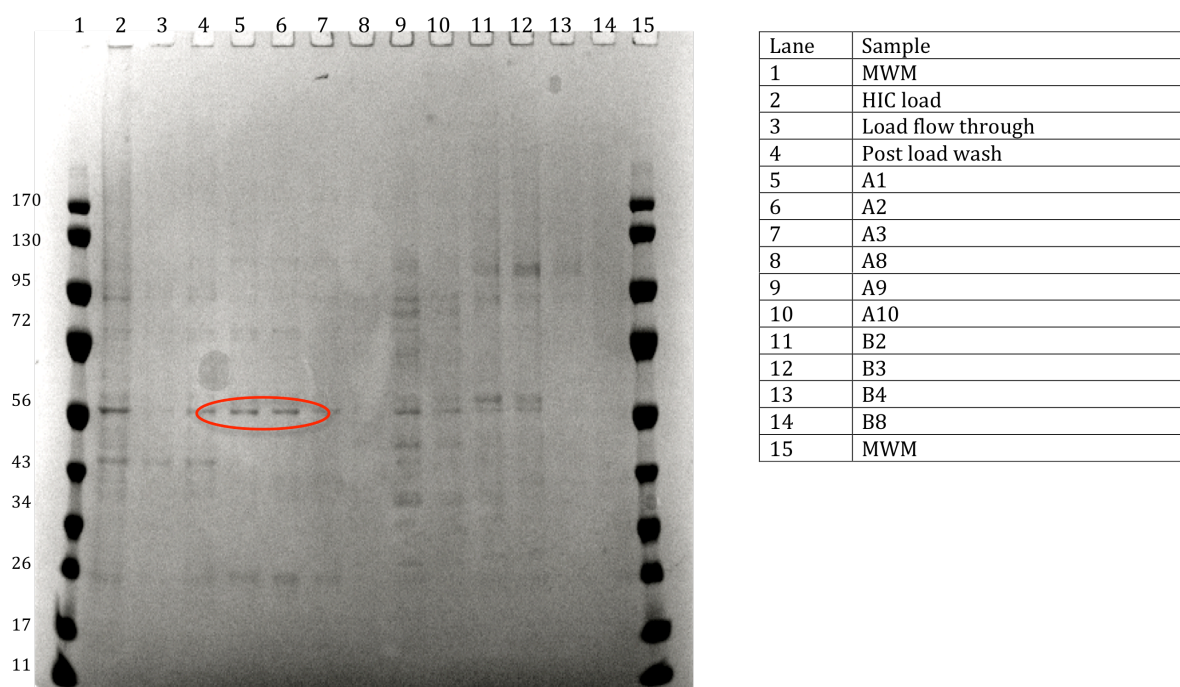


Figure 19. SDS-PAGE of hydrophobic interaction chromatography fractions for recombinant 5088 protein. The highlighted area indicates the location of putative purified 5088 protein in fractions A1 and A2 (lanes 5 and 6, respectively).

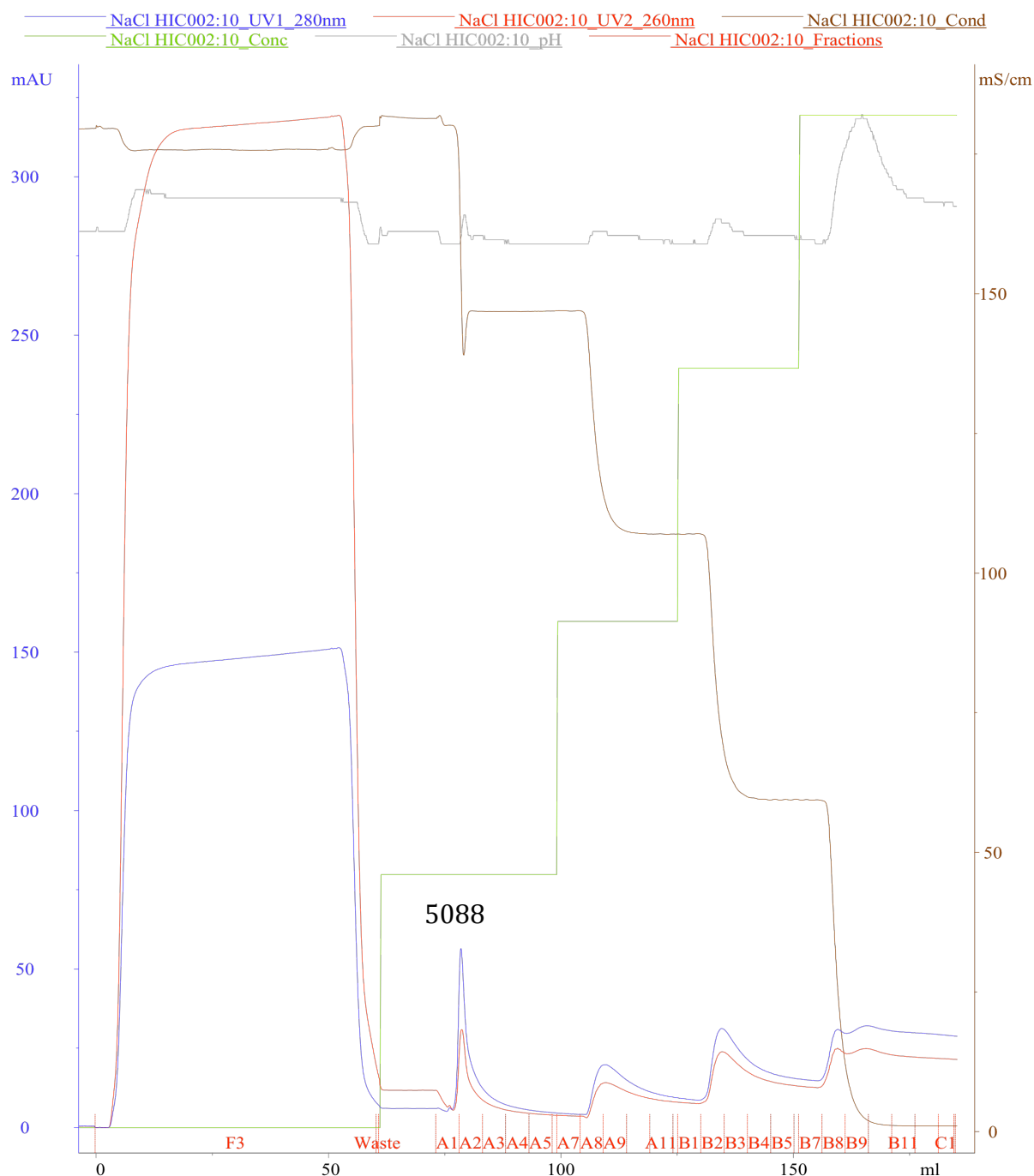


Figure 20. Chromatogram for hydrophobic interaction chromatography of recombinant 5088 protein. Putative purified 5088 protein was present in the first UV₂₈₀ peak following the loading step (fractions A1 and A2).

Table 4. Endotoxin levels of FA4000 and 5088 protein samples taken throughout the purification process.

Sample	FA4000 endotoxin (EU/ml)	5088 endotoxin (EU/ml)
Cell lysate	2×10^8	1.3×10^8
Refined lysate	1.5×10^6	5.1×10^7
Filtered aqueous phase	$2.5 \times 10^3 - 2 \times 10^4$	3.3×10^3
HIC fractions	36-170	106

Table 5. Total protein concentrations for FA4000 and 5088 recombinant protein samples taken during purification process.

Sample	FA4000 total protein concentration (mg/ml)	5088 total protein concentration (mg/ml)
cell lysate	13.9-18.3	15-17
preliminary spin supernatant	11.4-12.8	10.8-12.5
preliminary spin pellet	2.9-4	1.7-6.2
PEG supernatant	10.6-11.4	9.2-10.8
resuspended PEG pellet	0.9-1.6	1.2-5.2
Refined lysate	0.6-1.3	0.6-4.4
Refined lysate pellet	0.5-0.7	1.1-1.9
Aqueous phase	0.6-1	0.5-3.8
Detergent phase	0.4-0.5	0.3-0.7
HIC fractions	0.12-0.18	0.13-0.16

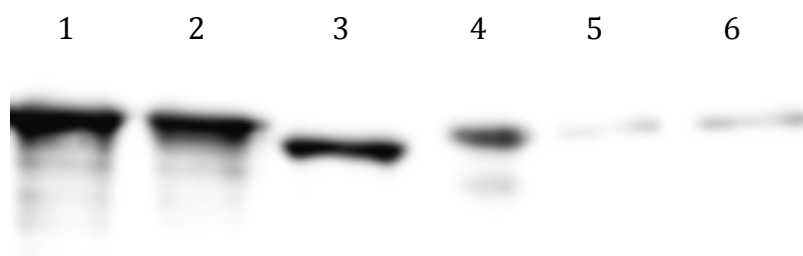


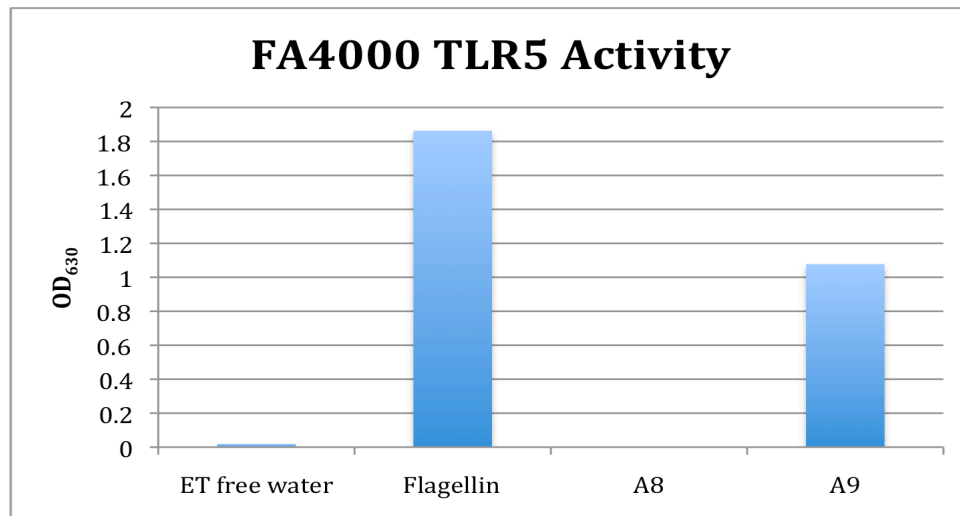
Figure 21. Image of Western Blot for detection of recombinant FliC. Lane 1 and 2, containing FA4000 fractions A8 and A9 respectively, and Lane 3, containing 5088, show the presence of FliC. Lanes 4-6 contain FA4000 purification samples from cells where induction of the FA4000 protein was low.

3.3. TLR5 Activity of purified recombinant FA4000 and 5088 proteins

Following the identification of FA4000 and 5088 proteins via Western Blot and mass spectrophotometry, the samples were tested for TLR5 activity to determine if the proteins were biologically active and able to induce an innate immune response. Preliminary results for the FA4000 and 5088 samples eluted directly from the HIC column showed either no TLR5 activity or lower activity than the positive control, commercially available recombinant *S. typhimurium* FliC (Figures 21A & B). The low TLR5 activity was determined to be due to the presence of high residual salt from the HIC separation. The high salt levels inhibited the growth of the HEK cells used in the TLR5 assay. The samples were desalted using a dextran desalting column (Pierce, Rockford, IL and GE Lifesciences, Piscataway, NJ). Figure 22 shows a typical desalting chromatogram, with the UV₂₈₀ peak indicating the protein has eluted followed by a separate peak in conductivity indicating the salt has eluted separately from the protein. The presence of FA4000 (Figure 23A & B) and 5088 (Figure 24) in fractions collected from the desalting column were confirmed using SDS-PAGE and silver staining to image the protein. Following desalting, the samples containing FA4000 and 5088 were re-tested for TLR5 activity and a significant increase in TLR5 activity was observed with the desalted protein (Figures 21b & 23b). TLR5 activity for FA4000 fraction A8 increased from 0% activity to 98% and fraction A9 increased from 58% activity to 97% (% activity was calculated using the activity of recombinant FliC as 100% activity). 5088 TLR5 activity increased from 0% activity to 115% activity in fraction A1 and 56% activity to 93% activity in fraction A2. The results indicate that the purified FA4000 and 5088 proteins are biologically active and capable of inducing an innate

immune response via TLR5. With the addition of a desalting step to the purification, the final purification scheme for soluble recombinant FA4000 and 5088 proteins is shown in Figure 27.

A.



B.

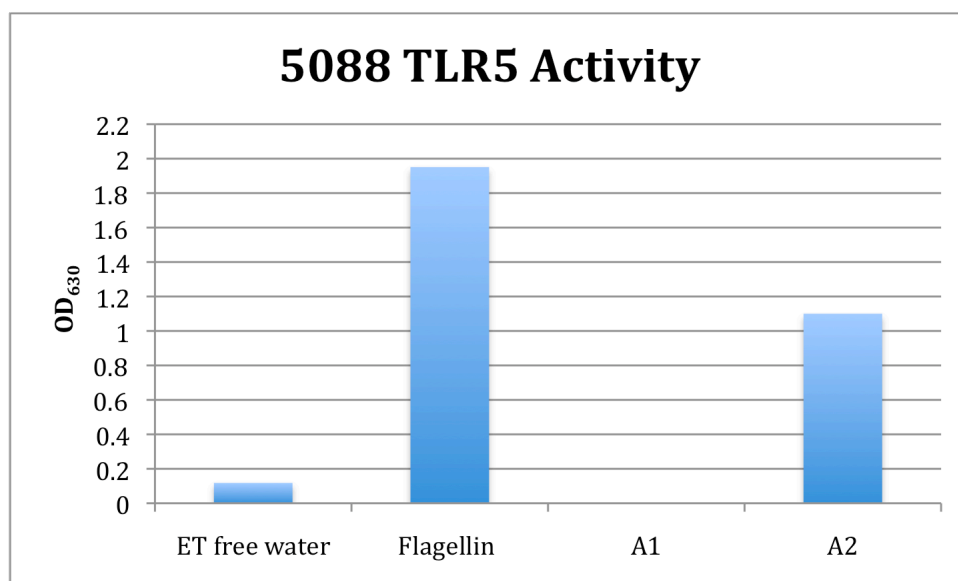


Figure 22. TLR5 activity of purified FA4000 and 5088 proteins following hydrophobic interaction chromatography A) TLR5 activity of purified FA4000, fractions A8 and A9 from HIC separation. B) TLR5 activity of purified 5088, fractions A1 and A2 from HIC separation.

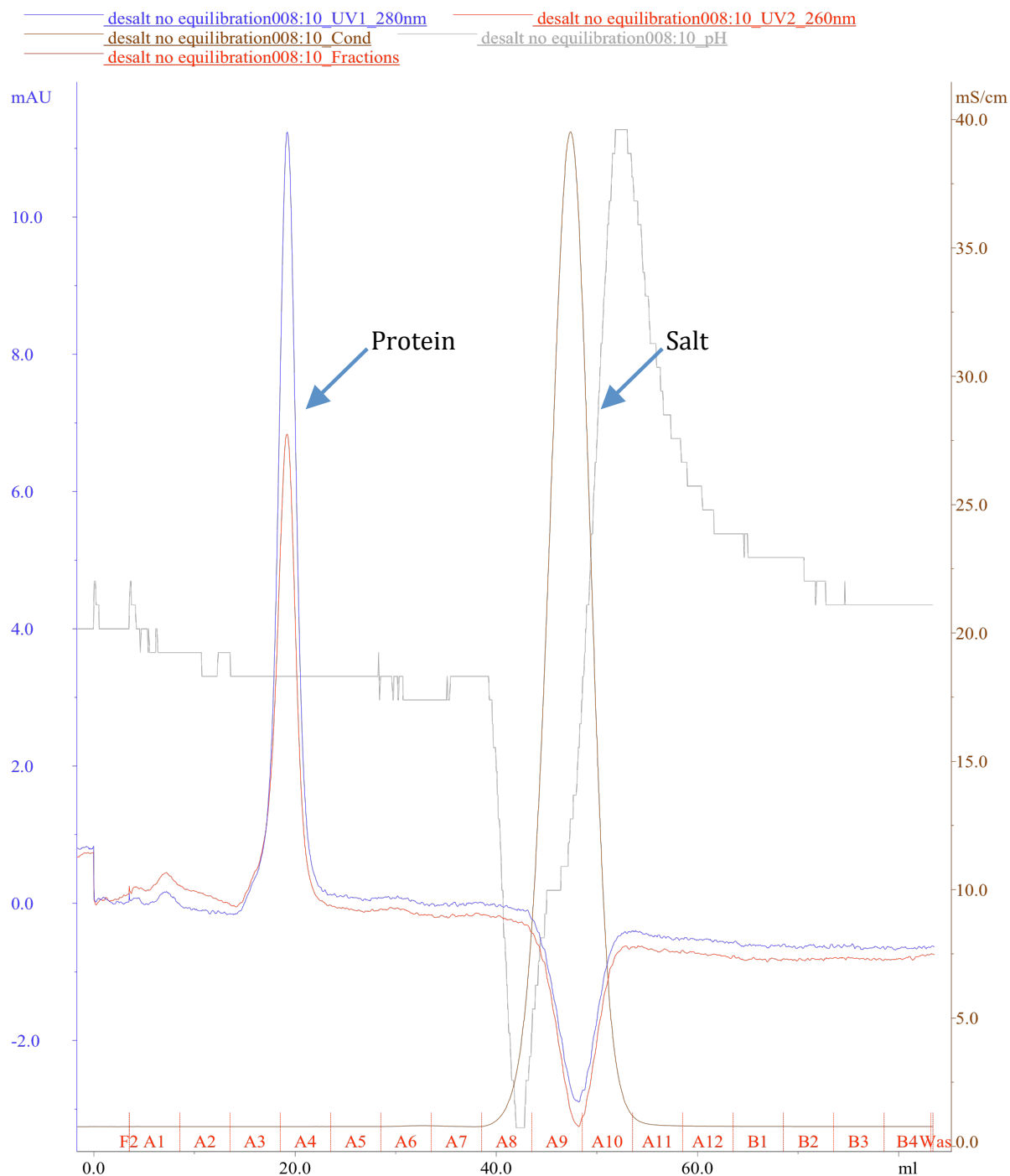


Figure 23. Chromatogram for protein desalting. The recombinant protein was present in fractions A3 and A4 indicated by a peak in the UV₂₈₀. The salt eluted later as indicated by a peak in the conductivity.

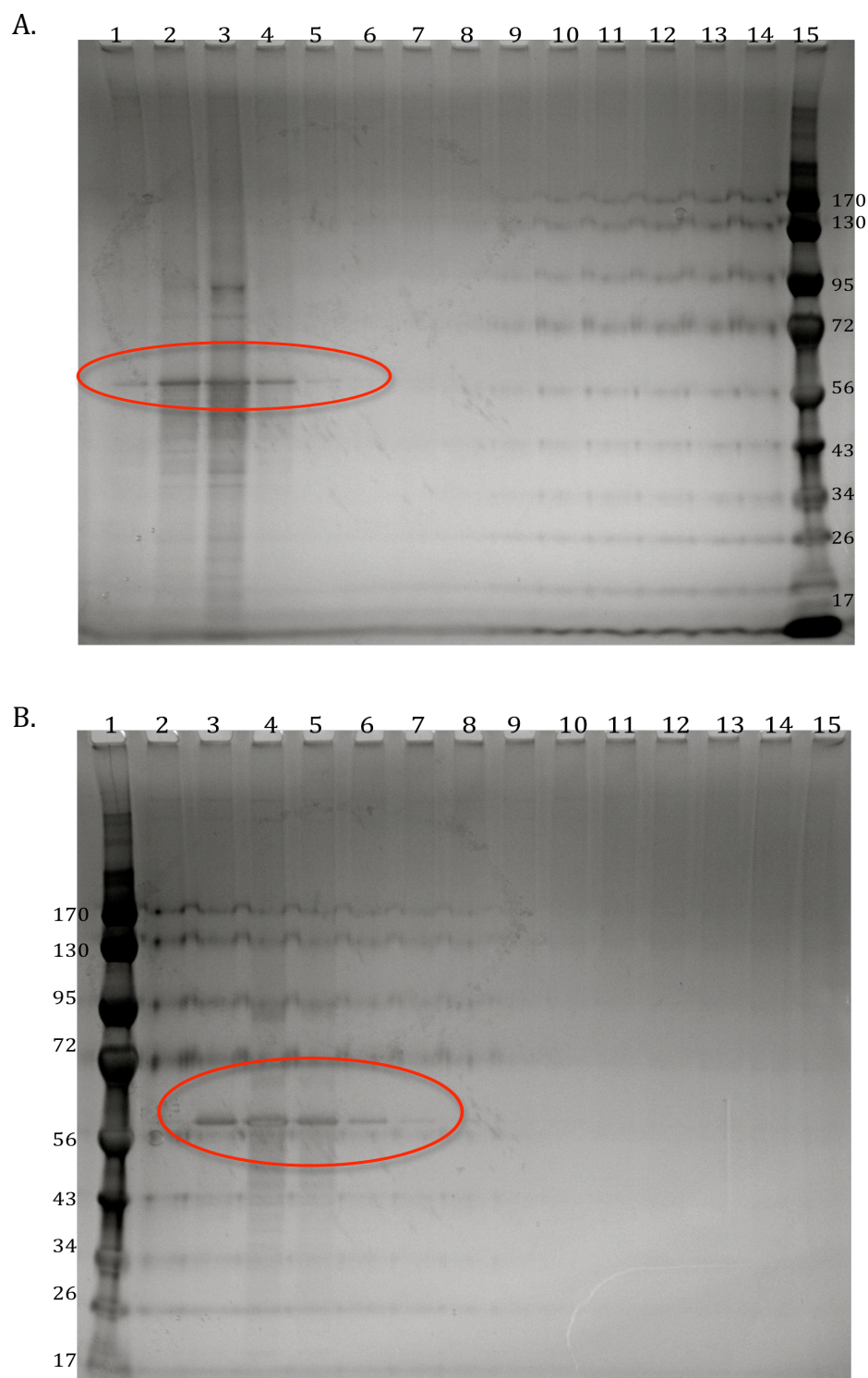


Figure 24. SDS-PAGE of FA4000 fractions collected from a desalting column. A) Silver stained SDS-PAGE of fraction A8 collected in 500 μ l fractions from 10ml desalting column. B) Silver stained SDS-PAGE gel of fraction A9 collected in 500 μ l fractions from 10ml desalting column. Highlighted areas indicate the location of the recombinant FA4000 protein.

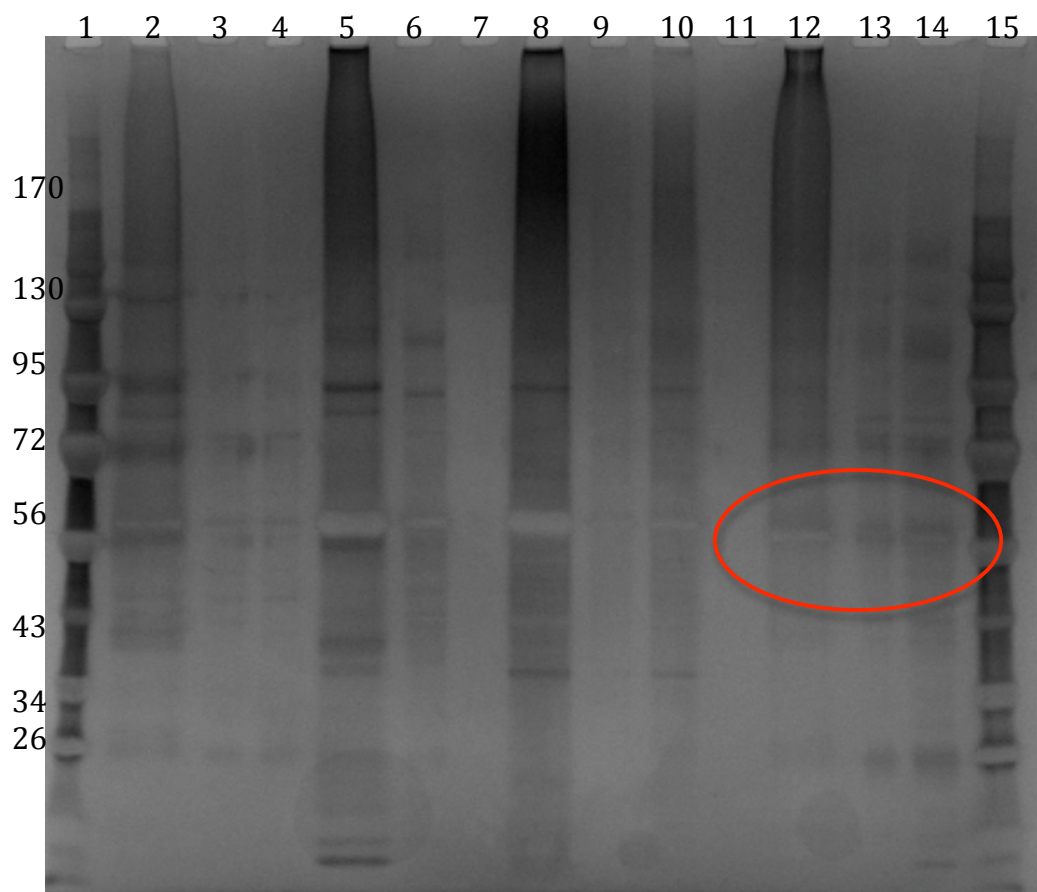
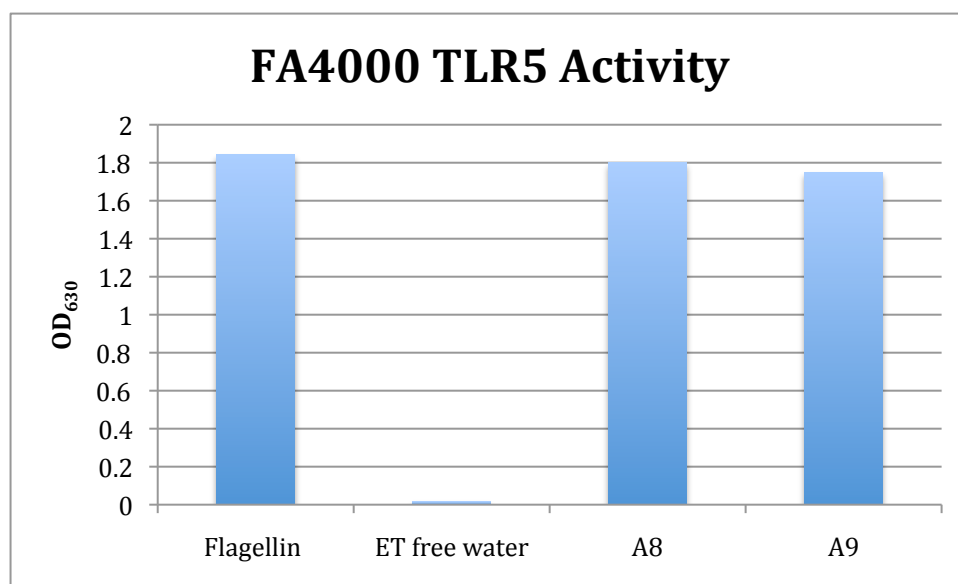


Figure 25. SDS-PAGE of 5088 fractions collected from a desalting column. Silver stained SDS-PAGE of 5088 fraction A1 and desalted 5088 protein in lanes 12-14. Highlighted area indicates the location of the recombinant 5088 protein. The protein bands are white due to overloading the gel.

A.



B.

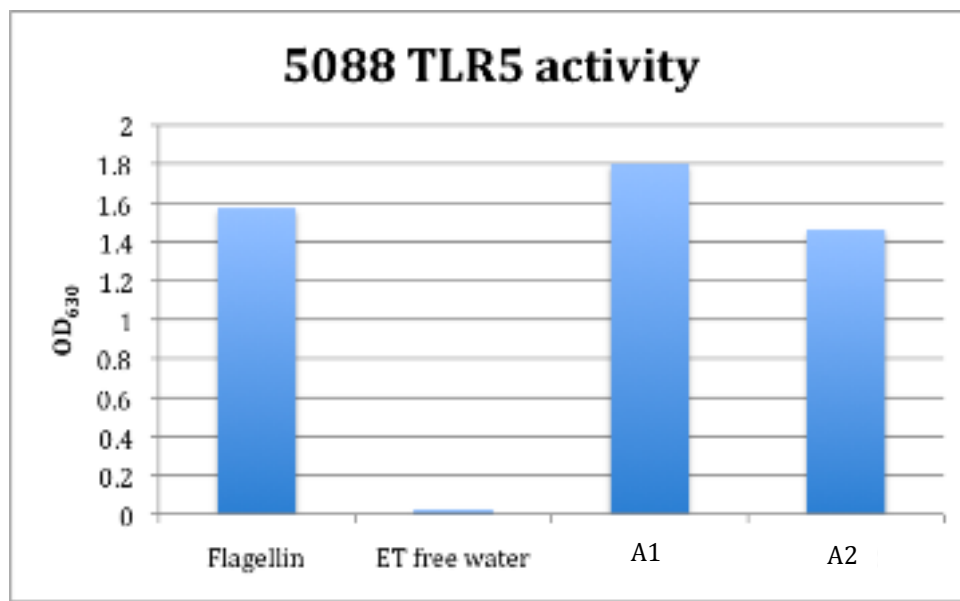


Figure 26. . TLR5 activity of purified FA4000 and 5088 proteins following removal of residual salt from hydrophobic interaction chromatography. A) TLR5 activity for recombinant FA4000 protein in fraction A8 and A9 following a desalting step. B) TLR5 activity for recombinant 5088 protein in fractions A1 and A2 following a desalting step.

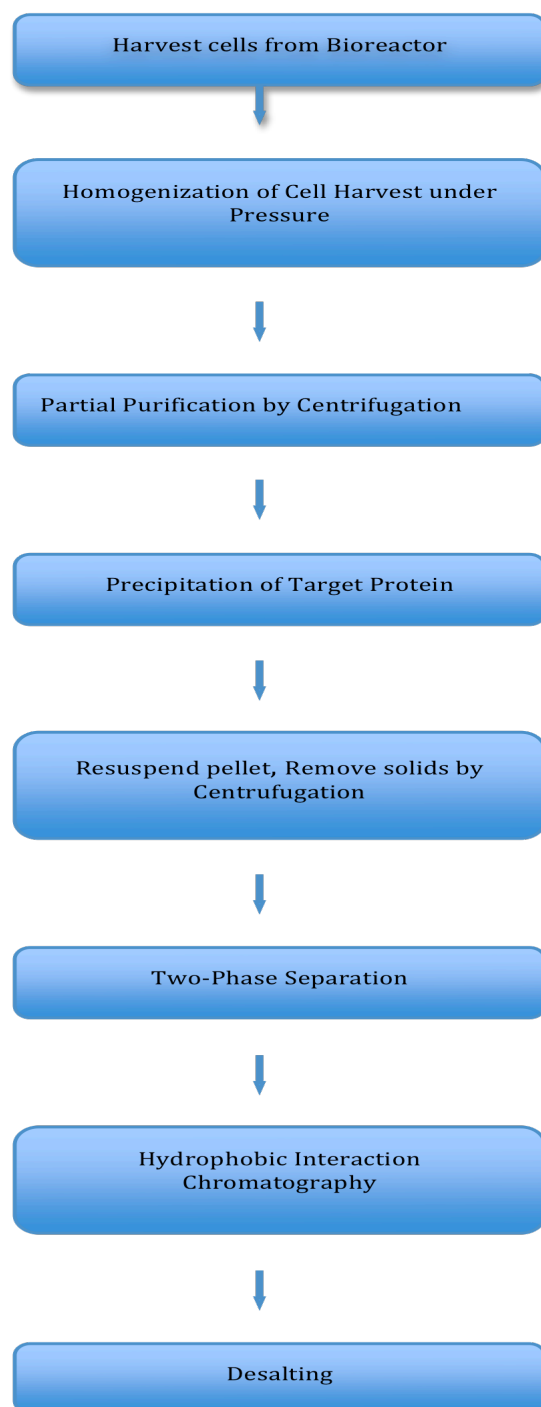


Figure 27. Purification strategy for soluble recombinant FliC constructs overexpressed in *E. coli*.

3.4. Comparison of TLR5 Activity for soluble recombinant protein and refolded recombinant protein

The TLR5 activity of purified soluble FA4000 and 5088 proteins was compared to the TLR5 activity of recombinant proteins that had been previously purified at GSU using a denaturing and refolding step. The TLR5 assay results showed that the soluble recombinant protein purified without using a denaturing and refolding step had a much higher TLR5 activity than recombinant protein that had been denatured and refolded (Figure 27). When compared to the TLR5 activity of the commercially available recombinant FliC standard, the TLR5 activity of recombinant protein that was denatured and refolded had 60-62% activity and soluble protein had 105-110% TLR5 activity. (% activity was calculated using the activity of recombinant FliC standard as 100% activity) The amount of refolded protein necessary to elicit a TLR5 response was also significantly higher than the amount of soluble protein. When compared to the recombinant FliC standard, the refolded protein required 35-700x the amount of protein to elicit a TLR5 response much lower than the FliC standard while the soluble protein required only 1.5-7.5x the amount of protein to elicit a TLR5 response comparable to the FliC standard (Table 7). Overall, these results indicate that soluble protein was more effective in inducing an innate immune response via TLR5 than recombinant protein that has been refolded.

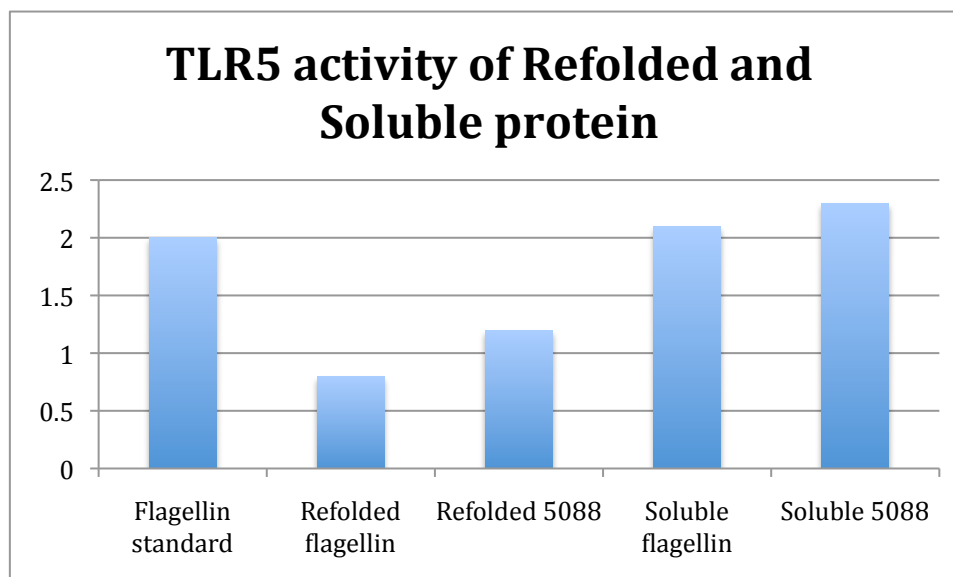


Figure 28. Comparison of TLR5 activity for refolded flagellin and 5088 proteins and soluble flagellin and 5088 proteins.

Table 6. TLR5 percent activity and protein concentrations of refolded and soluble proteins

Sample	% Activity	Protein concentration
Recombinant FliC Standard	100	0.2µg
Refolded Flagellin construct	62	7µg
Refolded 5088	60	140µg
Soluble FA4000	110	0.4-1.5µg
Soluble 5088	105	0.3-1µg

3.5. Stability of Recombinant FA4000 and 5088 recombinant protein at 4°C

Because the purified recombinant proteins are to be used as potential vaccines, it is important that they can be stored for a significant amount of time without losing their efficacy. Purified FA4000 and 5088 were stored at 4°C for a 6-month period and the TLR5 activity was tested to determine whether bioactivity was maintained during storage. Both the FA4000 and 5088 protein sustained TLR5 activity comparable to their initial activity following purification (Tables 8 & 9).

Table 7. TLR5 activity of FA4000 fractions A8 and A9 after 6 months of storage at 4°C. Chart shows % of TLR5 activity with positive control equal to 100% TLR5 activity.

Sample	% TLR5 activity
Fraction A8	97
Fraction A8 (stored at 4°C)	125
Fraction A9	95
Fraction A9 (stored at 4°C)	110

Table 8. TLR5 activity of 5088 fractions A1 and A2 after 6 months of storage at 4°C. Chart shows % of TLR5 activity with positive control equal to 100% TLR5 activity.

Sample	% TLR5 activity
Fraction A1	104
Fraction A1(stored at 4°C)	115
Fraction A2	105
Fraction A2(stored at 4°C)	93

4. DISCUSSION

In this study, purification of tag-less soluble recombinant full-length *Salmonella typhimurium* FliC flagellin (FA4000) and *Salmonella typhimurium* FliC flagellin with a Marberg virus antigen (GP132) linked at the C-terminal end (5088) proteins expressed in *E. coli* to vaccine standards was achieved utilizing well-established techniques that take advantage of the physical and chemical properties of the protein. The techniques and methods were appropriate for large-scale vaccine production and yielded enough recombinant protein for approximately 75,000-120,000 doses of vaccine in a single 30L fermentation. Previous research has shown protective immunity using flagellin fusion proteins in mice with doses as low as 1-3 μ g of recombinant protein (Honko et al., 2006; Song et al., 2008). Assuming a vaccine dose of 5 μ g, the endotoxin present in the vaccine would be 0.18-0.85EU/dose for FA4000 and 0.53EU/dose for 5088. Using the criteria of 5EU/kg bodyweight, the purified proteins fall well under this limit.

Using a purification strategy designed for only the soluble protein without steps to recover the insoluble protein, the time and potential cost necessary for purification were significantly reduced, primarily due to the removal of the tangential flow filtration, denaturing and refolding steps (Figure 28). The process of refolding recombinant protein into a biologically active molecule alone can account for a large portion of time and cost involved in commercial protein production (Datar et al., 1993). It was also hypothesized that by purifying the soluble protein without a denaturing and refolding step, the TLR5 activity for purified soluble recombinant protein would have a higher activity than recombinant protein that had been purified using denaturing and refolding steps. Soluble

protein that was purified using non-denaturing conditions and no refolding step, showed TLR5 activity comparable to 200ng of commercially available recombinant flagellin from *S. typhimurium* and required no more than 7.5x the amount of protein compared to the positive control. Protein purified using a refolding step showed TLR5 activity lower than the positive control for both recombinant 5088 and flagellin proteins and required 35x more protein for purified flagellin and 700x more protein for 5088 when compared to the amount of protein used as a positive control. This result was not surprising as typical yields of bioactive protein following protein refolding ranges from 15-40%, primarily due to protein aggregation during refolding (Singh and Panda, 2005; Yang et al., 2011).

Overall, the purification scheme used was effective in purifying both FA4000 and 5088 recombinant proteins to vaccine standards. However, some of the recombinant FA4000 and 5088 protein was lost during steps in purification, but this was expected as each purification step usually results in some protein loss and not all of the protein can be recovered without also picking up contaminants, especially during steps involving centrifugation. There was a significant loss of protein in the HIC step. A 10-15% protein yield was low, and both FA4000 and especially 5088 can be seen in the fractions that were not evaluated for this study. Future studies should address ways to increase the protein yield coming off of the HIC column by evaluating other types of HIC media and/or types of salt and salt concentrations used to dilute the protein prior to HIC.

While both FA4000 and 5088 proteins were purified using the same purification scheme there were notable differences with 5088. Hydrophobic interaction chromatography (HIC) was not as efficient in purifying 5088. There was 5088 protein lost in the column wash following the loading step, suggesting that some of the 5088 protein

did not bind well to the HIC column. In addition, there was also 5088 protein present in other protein peaks/fractions, which suggests the presence of 5088-dimer protein. The fractions from HIC also contained a higher level of contaminating protein indicating that HIC was not as efficient in separating 5088 protein from other contaminating proteins. 5088 also eluted earlier than FA4000 during the step gradient indicating that it is not as hydrophobic as FA4000, in spite of the fact that the protein sequences are very similar. Future studies should include the implementation of analytical testing to further characterize the recombinant flagellin (FliC) proteins.

Prior to utilizing HIC, anion exchange chromatography was evaluated for both FA4000 and 5088 using a strong anion exchange column, Toyopearl® Super Q 650M. The theoretical pI for FA4000 was 4.79 and for 5088 was 4.7 indicating that the overall protein charge at a pH of 8 would be negative which would make AEX suitable for use with the protein. However, neither protein bound efficiently to the AEX column and a majority of the recombinant protein passed through the column without binding to the media and was present in the load flow through. This might be due to endotoxin present in the sample. It is suggested that future experiments be conducted which evaluate an additional 2-phase separation to remove more endotoxin prior to AEX or performing the 2-phase separation before the PEG precipitation to improve binding of the target protein to the column. If anion exchange chromatography could be used instead of hydrophobic interaction, a desalting step wouldn't be necessary for purification, further reducing the purification time of the soluble protein.

The TLR5 activity of both FA4000 and 5088 samples tested following HIC were either much lower than expected or there was no activity at all. It was believed that the salt

concentration of the eluted protein was too high for TLR5 activity to be tested using the HEK-Blue™ TLR5 cells. A desalting step was evaluated to determine if the high salt concentration was interfering with the TLR5 assay. After desalting the samples using a HiPrep™ 26/10 desalting column, a significant increase in TLR5 activity was seen in all samples, confirming that the salt concentration following HIC was interfering with the HEK-Blue™ hTLR5 and null cells used for the TLR5 assay. A dextran column was chosen for desalting rather than dialysis because: 1) it is a much faster process and 2) can be scaled up for larger volumes by linking columns together in sequence, but can still be limiting for very large volumes. For much larger volumes it may be necessary to add a buffer exchange step using tangential flow filtration (TFF).

At present, most recombinant proteins containing flagellin are purified from insoluble protein or, if expressed as soluble protein, are usually his-tagged for ease of purification (Mizel and Bates, 2010). The results of this study indicate that recombinant FliC proteins expressed as soluble protein during a fermentation can be purified in an efficient and cost effective manner without the use of a tag and the purified soluble proteins exhibit a much higher TLR5 activity at a lower protein concentration than recombinant proteins that have been subjected to refolding. However, how recombinant proteins are expressed during fermentation is highly protein sequence dependent, suggesting that each recombinant protein will express differently, regardless of measures to push expression toward soluble protein (Basu et al., 2011). Currently, recombinant proteins expressed as inclusion bodies have been the most widely used for the commercial use of proteins (Walsh, 2003). This is due to the fact that most recombinant proteins,

approximately 70%, overexpressed in *E. coli* are insoluble protein (Yang et al., 2011). In order to optimize the yield of recombinant protein it is important to develop purification schemes that address both soluble and insoluble protein expression. While the purification of insoluble proteins may be easier with respect to removal of contaminants, refolding the protein into a bioactive molecule can often be time consuming and expensive. The insoluble protein must first be completely solubilized and then refolded into a biologically active protein creating a potential bottleneck in the purification process and this process often accounts for a major portion of the cost during production as there is no standardized method for refolding proteins. Even if future recombinant flagellin proteins are expressed primarily as insoluble, the purification scheme developed in this study could be used as a guide for purifying the soluble recombinant flagellin protein also present. Once purified, the TLR5 activity could be assayed to determine if the recombinant protein is biologically active while the solubilization and refolding of the insoluble protein is being optimized. Purification of the soluble recombinant flagellin protein in order to determine TLR5 activity could also prevent the investment of time and money into refolding the insoluble protein if the end product does not elicit an innate immune response via TLR5. This is especially important in recombinant flagellin proteins where portions of the native protein are being deleted and/or antigen is being inserted, as the deletions and/or insertions may effect the folding of the protein and its ability to induce a TRL5 immune response.

The hypotheses stated in the introduction were supported by this study. Both soluble recombinant FA4000 and 5088 were purified in less time and using fewer

steps than previously used in purification method A (Figure 10) that utilized a denaturing and refolding step to include both soluble and insoluble protein. The purified soluble recombinant proteins, FA4000 and 5088, also exhibited a much higher TLR5 activity at a lower concentration than recombinant proteins that had been refolded.

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7. APPENDICES

Appendix A. Detailed Laboratory Protocols

Homogenization

1. Thawing and Preparing Cells
 - a. Remove cells from -80°C freezer and record name of construct, fermentation date and amount of cell paste (g).
 - b. Place cells in 4°C refrigerator and allow to thaw overnight.
 - c. Retrieve thawed cells and chilled lysis buffer from 4°C refrigerator.
 - d. Calculate amount of lysis buffer needed to resuspend cell paste.

$$\text{Weight of cell paste (g)} / 0.15 = \text{amount of lysis buffer (ml)}$$

- e. Add a small amount of lysis buffer and stir bar to an appropriately sized container and then slowly pour thawed cells into the lysis buffer while stirring.
 - f. Rinse remaining cells from bottle using lysis buffer and add to container to be used for resuspending the cells.
 - g. Add remaining amount of lysis buffer to container.
 - h. Mix on stir plate for at least 20 minutes. Keep resuspended cells cold in an ice bath while stirring.
2. Prepare the APV-1000 homogenizer
 - a. While the cells are being resuspended, replace water in recirculating cooling loop with fresh cold ddH₂O and begin pumping (set flow to 50-100ml/min).
 - b. Fill hopper with 1.5L chilled ddH₂O and recirculate through APV for ≥ 10min and then drain.
 - c. Fill hopper with 500ml chilled lysis buffer and recirculate through APV for ≥ 10min, drain and discard lysis buffer.
 - d. Place container(s) for receiving homogenized cells into ice bath prior to beginning homogenization.
3. Homogenization
 - a. Sample re-suspended cells (2x1ml sample).
 - b. Load re-suspended cells into hopper. Homogenize for 1 pass through homogenizer at 0bar. Cool sample to 5-7°C before next pass in homogenizer
 - c. Homogenize sample for 3 more passes at 690 bar allowing the temperature to cool to 5-7°C prior to next pass. Monitor the temperature of the cell lysate as it leaves the homogenizer making sure the temperature of lysate stays below 24°C

- d. After the final pass, sample the cell lysate (2x1ml) and record the final weight and volume.
- e. If not processed immediately, store at 4°C.

4. Clean the Homogenizer

- a. Fill the hopper with 2L ddH₂O.
- b. Run through the homogenizer and discard.
- c. Fill the hopper with 2L fresh ddH₂O and recirculate for at least 10 minutes.
- d. Drain from homogenizer and discard.
- e. Fill hopper with 0.1N NaOH and recirculate for 10 minutes.
- f. Drain from homogenizer and discard.

Clarification by Centrifugation

1. Turn on centrifuge and set temperature to 4°C.
2. Aliquot equal volumes of lysate into centrifuge bottles and weigh each bottle to ensure that the centrifuge is balanced.
3. Load into centrifuge and spin for 1h @10,000rpm.
4. After centrifugation, collect supernatant via pipetting (pellet will be loose).
5. Pool the supernatants and measure total weight and volume.
6. Take sample of the pooled supernatant (2 x 1ml)
7. Pool pellets and weigh. Resuspend in Pellet Resuspension buffer equal to volume of supernatant. (May sample a smaller amount of the pellet and add equivalent amount of Pellet Resuspension Buffer)
8. Discard the pellet.

Polyethylene Glycol 3350 Protein Precipitation

1. Determine the amount of PEG 3350 needed for precipitation.

Amount of PEG 3350 to add= pooled supernatant volume (ml)*0.04 (4%)

2. Weigh out required amount of PEG 3350.
3. Slowly add the PEG 3350 to the pooled supernatant while stirring.
4. Once all of the PEG 3350 has been added and dissolved, allow to stir for ≥ 15 minutes.
5. Aliquot equal volumes of the pooled supernatant into centrifuge bottles and weigh each bottle to ensure that the centrifuge is balanced.
6. Load into centrifuge and spin for 1h @10,000rpm, 4°C.

7. After centrifugation, collect supernatant via pipetting or gently pouring. Measure volume of supernatant, sample (2 x 1ml) and discard. If not processed immediately, the PEG pellets can be stored at -20°C.

Pellet Resuspension and Centrifugation

1. Resuspend pooled PEG pellets in Resuspension Buffer (half the volume of the supernatant from previous centrifugation)
2. Disrupt pellets mechanically using a disposable inoculation needle.
3. Mix pellets with a stir bar until the pellet has been completely resuspended.
4. Sample the resuspended pellet (2x1ml).
8. Aliquot equal volumes of the resuspended pellet into centrifuge bottles and weigh each bottle to ensure that the centrifuge is balanced.
9. Load into centrifuge and spin for 1h @10,000rpm, 4°C.
10. After centrifugation collect supernatant via pipet, pool supernatants and record total volume and weight. Collect 2x1ml samples. This is the "refined lysate."
11. If not processed immediately, store Refined lysate at 4°C.
12. Pool pellets and record weight. Resuspend in Pellet Resuspension buffer equal to volume of the Refined lysate (May sample a smaller amount of the pellet and add equivalent amount of Pellet Resuspension Buffer).
13. Discard the pellet

2-phase separation

1. Calculate the amount of Triton® X-114 (1% (w/w) to add to Refined lysate.

Weight of Refined lysate (mg)*0.01= amount of Triton X®-114 to add

2. Add calculated amount of Triton X®-114 to Refined lysate
3. Stir for 30 minutes.
4. Aliquot equal volumes of the Refined lysate into centrifuge tubes and weigh each tube to ensure that the centrifuge is balanced.
5. Centrifuge for 4h @ 10000rpm, 24°C (Decel set to 0). Sample can be left overnight in centrifuge.
6. Carefully retrieve the samples from the centrifuge.
7. Gently pipet off the top layer (aqueous phase) of the 2-phase separation.
8. Pool the aqueous phase from each centrifuge tube and record the total volume. Sample the aqueous phase (2 x 1ml).
9. Filter using a 0.2
10. If not immediately processed, store at 4°C.
11. Pool the bottom layer (detergent phase), record volume, sample (2 x 1ml) and discard.

Lysis Buffer - 20mM Tris, 100mM NaCl, 1mM EDTA, 100mM sucrose, pH 8 (1L)

- Add 750ml ddH₂O to a 1L bottle with a stir bar.
- Add 34.2g of sucrose.
- Add 2.42g of tris
- Add 5.82g of NaCl
- Add 372mg of EDTA
- Stir until completely dissolved.
- Adjust pH using Acetic Acid
- QS to 1L with ddH₂O.
- Filter sterilize, (0.2 µm filter unit).
- Store at 4°C

Resuspension Buffer - 20mM Tris, 1mM EDTA, 100mM sucrose, pH 8 (1L)

- Add 800 ml ddH₂O to a 1L bottle with a stir bar
- Add 2.42g of Tris
- Add 372mg of EDTA
- Add 34.2g of sucrose
- Stir until completely dissolved
- Adjust pH using acetic acid
- QS to 1L with ddH₂O.
- Filter sterilize, (0.2 µm filter unit)
- Store at 4°C

Pellet Resuspension Buffer – 20mM tris, 8M urea, pH 8

- Add 500ml ddH₂O to a 1L bottle with a stir bar.
- Gradually add 480.48g of Urea.
- Stir until completely dissolved.
- Add 2.42g of Tris.
- Stir until completely dissolved.
- Adjust pH to 8 with acetic acid.
- Make up to 1L with ddH₂O ..
- Filter sterilize, (0.22µm CA or PES filter unit)
- Store at room temperature

Hydrophobic Interaction Chromatography

1. Sample preparation
 - a. Calculate volume of sample containing 15mg total protein and add to 250ml container.
 - b. While stirring, slowly add an equal volume of Dilution Buffer (20mM tris, 5M NaCl, pH8) and allow to stir for at least 10 minutes following addition of Dilution Buffer.
 - c. Filter samples using a 0.2µm PES syringe filter.
 - d. Dilute sample prior to loading onto column to a final protein concentration of 0.2mg/ml with Elution Buffer (20mM tris, 2.5M NaCl, pH 8).
2. ÄKTAexplorer™ 100 preparation
 - a. Prior to beginning HIC program, prime the system buffer lines to be used for the program.
 - b. Prime the sample line, S1, with equilibration buffer and leave the line in equilibration buffer until just before the program is started.
 - c. Remove pH meter from storage buffer and rinse with ddH₂O. Remove “dummy” pH meter and replace with pH meter.
3. Hydrophobic Interaction Chromatography
 (Chromatography was performed using the ÄKTAexplorer™ 100 system and UNICORN 5.01 control software (GE Lifesciences, Piscataway, NJ) and the method created using Method Wizard.)
 Flow rate for program was 5ml/min
 - a. Place Equilibration Buffer/Elution Buffer 1 on Buffer line assigned during the Method Wizard set up. (Buffer line A11- A18)
 - b. Place Equilibration Buffer 2 on Buffer line B1.
 - c. Equilibrate column with 8CV of Equilibration Buffer (20mM tris, 2.5M NaCl, pH 8).
 - d. Load 10mg (50ml) of protein onto HIC column. Manually collect load flow through from Outlet line F3 in a 50ml centrifuge tube.
 - e. Wash column with 2CV of Equilibration Buffer (20mM tris, 2.5M NaCl, pH 8) once all protein has been loaded to the column. Manually collect flow through from Outlet line F3 in a 15ml centrifuge tube.
 - f. Elute bound protein with a step gradient of 25%, 50%, 75% and 100% Elution Buffer 2 (20mM tris, pH 8). Collect in 5ml fractions using Frac-950, outlet F2.

Dilution Buffer – 5M NaCl, 20mM tris, pH 8 (1L)

- Add 700ml ddH₂O to a 1L bottle with a stir bar
- Add 292.2g NaCl
- Add 2.42g tris
- Stir for ≥ 10 min
- Adjust pH using acetic acid
- QS to 1L with ddH₂O.
- Filter sterilize, (0.2 μ m filter unit)
- Store at room temp

Equilibration Buffer/Elution buffer 1– 2.5M NaCl, 20mM tris, pH 8 (1L)

- Add 700ml ddH₂O to a 1L bottle with a stir bar
- Add 146.1g NaCl
- Add 2.42g tris
- Stir for ≥ 10 min
- Adjust pH using acetic acid
- QS to 1L with ddH₂O.
- Filter sterilize, (0.2 μ m filter unit)
- Store at room temp

Elution Buffer 2 - 20mM Tris, pH 8.0 (1L)

- Add 900 ml ddH₂O to a 1L bottle with a stir bar
- Add 2.42g of Tris
- Stir for ≥ 10 min
- Adjust pH using acetic acid
- QS to 1L with ddH₂O.
- Filter sterilize, (0.2 μ m filter unit)
- Store at room temp

TLR5 Assay

Day 1

1. Prepare test medium (same as growth medium)

DMEM, 4.5g/l glucose
 50ul/ml penicillin
 50ug/ml streptomycin
 100ug/ml Normocin,
 2mM L-glutamine
 10% (v/v) heat-inactivated FBS

2. Prepare cell suspension of HEK-blue-hTLR5 cells and null cells of ~140,000 cells/ml in test medium

Counting HEK cells with Neubauer hemocytometer

- a. Count the cells in the 4 large corner squares and the large middle square (each large square is made up of 16 little squares)
 - b. Add the total cell count (must be >than 100 total)
 - c. Calculating cell #

$$(\text{Total \# cells counted} / 0.5\text{mm}^3) (1000) (\text{dilution factor}) = \text{\#cells/ml}$$
3. Add 20ul of each sample per well of a flat bottom 96 well plate
 4. Add 20ul of RecFLA-ST (flagellin) in one well as a positive control and 20ul of sterile, endotoxin free water in another well as a negative control
 5. Add 180ul of cell suspension/well
 6. Incubate plate at 37C in a CO2 incubator for 20-24 hours.

Day 2

1. Prepare QUANTI-Blue solution
2. Add 180ul of resuspended QUANTI-Blue per well of a 96-well plate.
3. Incubate the plate at 37C incubator for 1-3h.
4. Determine SEAP levels using a spectrophotometer at 620-655λ

Reviving TLR5 cells from glycerol stock

1. Before using the tissue culture hood, clean the hood with 70% ethanol.
2. Remove glycerol stocks from nitrogen storage and place in 37°C water bath to thaw.
3. While glycerol stocks are thawing, add required amount of growth media w/o antibiotics to tissue culture flask, place in CO₂ incubator and allow to warm media
4. When glycerol stocks have thawed remove glycerol stocks from water bath and ethanol the cryovials before placing in the hood.
5. Remove the growth media from the CO₂ incubator, ethanol and place in the hood.
6. Add HEK cells (hTLR5 and Null) to the tissue culture flasks containing growth media and label flasks.
7. Place in 37°C, 5% CO₂ incubator

Growth media for HEK hTLR5 and Null cells (w/o antibiotics)

- Remove 58.5ml of media from DMEM with 4.5g/L NaCl (Corning, Tewksbury, MA)
- Add 50ml Heat inactivated Fetal Bovine Serum (Invitrogen, Grand Island, NY)
- Add 2.5ml concentrated Penicillin/Streptomycin solution (Corning, Tewksbury, MA)
- Add 5ml 200mM glutamine solution (Corning, Tewksbury, MA)
- Add 1ml Normocin™ (50mg/ml, Invivogen, San Diego, CA)

Test media for hTLR5 cells (w/ antibiotics)

- Place 50ml of growth media w/o antibiotics into a 50ml falcon tube
- Add 50ul Zeocin™ (100mg/ml in HEPES buffer, Invivogen, San Diego, CA)
- Add 150ul Blasticidin (10mg/ml in HEPES buffer, Invivogen, San Diego, CA)

Test media for Null cells (w/ antibiotics)

- Place 50ml of growth media without antibiotics into a 50ml falcon tube
- Add 50ul of Zeocin™ (100mg/ml in HEPES buffer, Invivogen, San Diego, CA)

Appendix B. Sampling and Testing Schedule

Process Step	Sample	BCA	SDS-PAGE	Endotoxin	Western Blot (FliC)
Homogenization	Resuspended cell paste		x		
Homogenization	lysate (final pass)	x	x	x	
Initial spin	pooled supernatant	x	x		
Initial spin	resuspended pellet	x	x		
PEG precipitation	pooled supernatant	x	x		
Resuspension	resuspended pellet	x	x		
Refined Lysate	RL supernatant	x	x	x	
Refined Lysate	post spin resuspended pellet	x	x		
2-phase	Aqueous phase	x	x		
2-phase	Detergent phase	x	x		
2-phase	Filtered aqueous phase	x	x	x	
HIC	HIC load	x	x		
HIC	Flow through (FT)	x	x		
HIC	Wash	x	x		
HIC	Peak Fractions	x	x	x	x
HIC	Strip	x	x		

Appendix C. Mass Spectrophotometry Results

5088

Gel Idx/Pos		106/E5		Instr./Gel Origin		ak167/6616-061214		Process Status		Analysis Succeeded	
Plate #/ Name		[1] 6616-061214-kyu		Instrument Sample Name				Spectra		11	
Rank	Protein Name	Accession No.	Protein MW	Protein PI	Total Ion Score	Total Ion C. I. %	Pep. Species Count	Molecular Function			
1	flagellin [alternatively spliced] [Salmonella typhimurium, SJW61, Peptide Mutant, 405 aa]	gi 834003	42513.6	4.97	154	100	3				
<div>Protein Group</div>											
	Chain A, Crystal Structure Of F41 Fragment Of Flagellin	gi 13786773	41292.7	4.6500							
				000953							
				6743							
	flagellin - Salmonella typhimurium	gi 96744	51450	4.7899							
				999618							
				5303							
	flagellin [alternatively spliced] [Salmonella typhimurium, SJW46, Peptide Mutant, 397 aa]	gi 834004	41725.2	4.9800							
				000190							
				7349							
	phase 1 flagellin [Salmonella enterica subsp. enterica serovar Heidelberg]	gi 50830946	51265.9	4.8200							
				001716							
				6138							
	phase 1 flagellin [Salmonella enterica subsp. enterica serovar Heidelberg]	gi 50830950	51610.1	4.8800							
				001144							
				4092							
	phase 1 flagellin [Salmonella enterica subsp. enterica serovar Kinshasa]	gi 50830934	51934.3	4.9600							
				000381							
				4697							
	phase 1 flagellin [Salmonella enterica subsp. enterica serovar Typhimurium]	gi 50830924	51580.1	4.8800							
				001144							
				4092							
	phase 1 flagellin [Salmonella enterica subsp. enterica serovar Typhimurium]	gi 50830926	51581	4.7899							
				999618							
				5303							
	phase 1 flagellin [Salmonella enterica]	gi 38049925	52139.3	4.8099							
				999427							

Gel Idx/Pos Plate [#] Name	130/F5 [1] 6616-061214-kyu	Instr./Gel Origin Instrument Sample Name	ak167/6616-061214	Process Status Spectra	Analysis Succeeded 11			
Rank	Protein Name	Accession No.	Protein MW	Protein PI	Total Ion Score	Total Ion C. I. %	Pep. Species Count	Molecular Function
1	flagellin [alternatively spliced] [Salmonella typhimurium, SJW61, Peptide Mutant, 405 aa]	gjl834003	42513.6	4.97	97	100	2	
<div>Protein Group</div> <div>Chain A, Crystal Structure Of F41 Fragment Of Flagellin</div>								
	flagellin - Salmonella typhimurium	gjl96744	41292.7	4.6500 000953 6743				
	flagellin [alternatively spliced] [Salmonella typhimurium, SJW46, Peptide Mutant, 397 aa]	gjl834004	41725.2	4.7899 999618 5303				
	phase 1 flagellin [Salmonella enterica subsp. enterica serovar Heidelberg]	gjl50830946	51265.9	4.8200 001716 6138				
	phase 1 flagellin [Salmonella enterica subsp. enterica serovar Heidelberg]	gjl50830950	51610.1	4.8800 001144 4092				
	phase 1 flagellin [Salmonella enterica subsp. enterica serovar Kinshasa]	gjl50830934	51934.3	4.9600 000381 4697				
	phase 1 flagellin [Salmonella enterica subsp. enterica serovar Typhimurium]	gjl50830924	51580.1	4.8800 001144 4092				
	phase 1 flagellin [Salmonella enterica subsp. enterica serovar Typhimurium]	gjl50830926	51581	4.7899 999618 5303				
	phase 1 flagellin [Salmonella enterica]	gjl38049925	52139.3	4.8099 999427 7954				
	phase 1 flagellin [Salmonella enterica]	gjl38050029	51639.1	4.75				
	phase 1 flagellin [Salmonella enterica]	gjl38050051	51519	4.8299 999237 0605				
	phase 1 flagellin [Salmonella enterica]	gjl38050061	51518.1	4.8899 998664 856				
	phase 1 flagellin [Salmonella enterica]	gjl38050077	51941.4	5.0399 999618 5303				
	phase 1 flagellin [Salmonella enterica]	gjl38050113	51167.8	4.8299 999237 0605				
	phase 1 flagellin [Salmonella enterica]	gjl38050133	52790.7	4.9099				

	Peptide Information						Rank Result Type
	Calc. Mass	Obsrv. Mass	\pm da	\pm ppm	Start Seq.	End Sequence	
phase 1 flagellin [Salmonella enterica]							
	2007.0254	2006.9974	-0.028	-14	343	361 FNSAITNLGNTVNNLTSA	1 Mascot
phase 2 flagellin [Salmonella enterica]							
	2670.3076	2670.2783	-0.0293	-11	66	90 NANDGISIAQTTEGALNEI	1 Mascot
phase 2 flagellin [Salmonella enterica]						NNLOR	
phase 2 flagellin [Salmonella enterica]		2805.3496	-0.0173	-6	93	118 ELAYQSANSTNSQSDLD	1 Mascot
phase 2 flagellin [Salmonella enterica]						SIOAEITQR	
2 phase 1 flagellin [Salmonella enterica]					gj 46359071	51430.9	3
						4.89	100
						154	
						4.89	
						51386	
						4.9899	
						997711	
						1816	
						4.7699	
						99809	
						2651	
						4.9899	
						997711	
						1816	
						4.9899	
						998092	
						6514	
						4.9899	
						997711	
						1816	
						4.7699	
						99809	
						2651	
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						998092	
						6514	
						4.9899	
						997711	
						1816	
						4.7699	
						99809	
						2651	

Peptide Information									
Calc. Mass	Obsrv. Mass	± da	± ppm	Start Seq.	End Sequence Seq.	Ion Score	C. I. % Modification	Rank	Result Type
2007.0254	2006.9974	-0.028	-14	433	451 FNSAITNLGNTVXNL TSA	51	99.984	1	Mascot
2670.3076	2670.2783	-0.0293	-11	67	91 NANGISIAQTTEGALNEI	58	99.997	1	Mascot
2805.3496	2805.3323	-0.0173	-6	94	119 ELAVQSANSTNSQSDLD	46	99.951	1	Mascot
3 phase 2 flagellin [Salmonella enterica]									
				gi 38049874	51967.1	4.95	100	3	
Peptide Information									
Calc. Mass	Obsrv. Mass	± da	± ppm	Start Seq.	End Sequence Seq.	Ion Score	C. I. % Modification	Rank	Result Type
2007.0254	2006.9974	-0.028	-14	433	451 FNSAITNLGNTVNNL TSA	51	99.984	1	Mascot
2670.3076	2670.2783	-0.0293	-11	67	91 NANGISIAQTTEGALNEI	58	99.997	1	Mascot
2805.3496	2805.3323	-0.0173	-6	94	119 ELAVQSANSTNSQSDLD	46	99.951	1	Mascot

Gel Idx/Pos	130/F5	Instr./Gel Origin	ak167/6616-061214	Process Status	Analysis Succeeded			
Plate [#] Name	[1] 6616-061214-kyu	Instrument Sample Name		Spectra	11			
Rank	Protein Name	Accession No.	Protein MW	Protein PI	Total Ion Score	Total Ion C. I. %	Pep. Species Count	Molecular Function
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Protein Group								
	Chain A, Crystal Structure Of F41 Fragment Of Flagellin	gi 13786773	41292.7	4.6500				
				000953				
				6743				
	flagellin - Salmonella typhimurium	gi 96744	51450	4.7899				
				999618				
				5303				
	flagellin {alternatively spliced} [Salmonella typhimurium, SJW46, Peptide Mutant, 397 aa]	gi 834004	41725.2	4.9800				
				000190				
				7349				
	phase 1 flagellin [Salmonella enterica subsp. enterica serovar Heidelberg]	gi 50830946	51265.9	4.8200				
				001716				
				6138				
	phase 1 flagellin [Salmonella enterica subsp. enterica serovar Heidelberg]	gi 50830950	51610.1	4.8800				
				001144				
				4092				
	phase 1 flagellin [Salmonella enterica subsp. enterica serovar Kinshasa]	gi 50830934	51934.3	4.9600				
				000381				
				4697				
	phase 1 flagellin [Salmonella enterica subsp. enterica serovar Typhimurium]	gi 50830924	51580.1	4.8800				
				001144				
				4092				
	phase 1 flagellin [Salmonella enterica subsp. enterica serovar Typhimurium]	gi 50830926	51581	4.7899				
				999618				
				5303				
	phase 1 flagellin [Salmonella enterica]	gi 38049925	52139.3	4.8099				
				999427				
				7954				
	phase 1 flagellin [Salmonella enterica]	gi 38050029	51639.1	4.75				
	phase 1 flagellin [Salmonella enterica]	gi 38050051	51519	4.8299				
				999237				
				0605				
	phase 1 flagellin [Salmonella enterica]	gi 38050061	51518.1	4.8899				
				998664				
				856				
	phase 1 flagellin [Salmonella enterica]	gi 38050077	51941.4	5.0399				
				999618				
				5303				
	phase 1 flagellin [Salmonella enterica]	gi 38050113	51167.8	4.8299				
				999237				
				0605				
	phase 1 flagellin [Salmonella enterica]	gi 38050133	52790.7	4.9099				

phase 1 flagellin [Salmonella enterica]	gjl38050271	51492	998474 1211 4.9600 000381 4697 4.8400 001525 8789 4.75
phase 1 flagellin [Salmonella enterica]	gjl46359069	51486.1	
phase 1 flagellin [Salmonella enterica]	gjl38050243	52697.6	
phase 1 flagellin [Salmonella enterica]	gjl38050239	52564.5	4.8699 998855 5908
phase 1 flagellin [Salmonella enterica]	gjl38050225	52459.5	4.8800 001144 4092
phase 1 flagellin [Salmonella enterica]	gjl38050221	52190.6	5.0399 999618 5303
phase 1 flagellin [Salmonella enterica]	gjl38050219	52190.5	4.9699 997901 9165
phase 1 flagellin [Salmonella enterica]	gjl38050215	52045.3	4.9899 997711 1816
phase 1 flagellin [Salmonella enterica]	gjl38050147	52146.3	4.8099 999427 7954
phase 1 flagellin [Salmonella enterica]	gjl38050115	51153.8	4.8200 001716 6138
phase 1 flagellin [Salmonella enterica]	gjl38050109	51251.9	4.8200 001716 6138
phase 1 flagellin [Salmonella enterica]	gjl38050067	51510	4.8400 001525 8789
phase 1 flagellin [Salmonella enterica]	gjl38050059	51469.1	4.9499 998092 6514
phase 1 flagellin [Salmonella enterica]	gjl38050037	51489	4.8299 999237 0605
phase 2 flagellin [Salmonella enterica]	gjl38049788	52156.3	4.9699 997901 9165
phase 2 flagellin [Salmonella enterica]	gjl38049798	52092.3	5.0100 002288 8184
phase 2 flagellin [Salmonella enterica]	gjl38049804	52090.3	5.0100 002288 8184
phase 2 flagellin [Salmonella enterica]	gjl38049768	52550.5	4.8600 001335

2007.0254	2007.0262	0.0008	0	433	451	FNSAITNLGNTVNNLTSA	32	99.022	1	Mascot
2870.3076	2870.3127	0.0051	2	67	91	R NANDGISIAQTTEGALNE XNNLQR	65	99.999	1	Mascot

Gel Idx/Pos		11/A11		Instr./Gel Origin		ak167/6616-083112		Process Status		Analysis Succeeded	
Plate [#] Name		[1] 6616-083112		Instrument Sample Name				Spectra		13	
Rank	Protein Name	Accession No.	Protein MW	Protein PI	Total Ion Score	Total Ion C. I. %	Pep. Species Count	Molecular Function			
1	phase 2 flagellin [Salmonella enterica]	gi 38049874	51967.1	4.95	1241	100	9				
Peptide Information											
	Calc. Mass	Obsrv. Mass	± da	± ppm	Start Seq.	End Sequence	Ion Score	C. I. %	Modification	Rank Result Type	
	959.4904	959.4675	-0.0229	-24	424	432 SDLGAVQNR	57	99.997		1 Mascot	
	1132.5957	1132.5677	-0.028	-25	21	31 SQSALGTAIER	75	100		1 Mascot	
	1285.7111	1285.6799	-0.0312	-24	412	423 IDAALAQVDTLR	109	100		1 Mascot	
	1614.8195	1614.7792	-0.0403	-25	38	53 INSAKDDAAGQAANR	170	100		1 Mascot	
	2007.0254	2006.9684	-0.057	-28	433	451 FNSAITNLGNTVNLTSAR	160	100		1 Mascot	
	2671.2666	2671.2273	-0.0393	-15	67	91 NANDGISIAQTTEGALNE XNNLQR	157	100		1 Mascot	
	2805.3496	2805.2722	-0.0774	-28	94	119 ELAVQSANSTNSQSDLD SIQAEITQR	204	100		1 Mascot	
	2860.6003	2860.511	-0.0893	-31	469	495 AQILQQAGTSVLAQANQ VPQNVLSLR	180	100		1 Mascot	
	3060.5193	3060.4424	-0.0769	-25	92	119 VRELAVQSANSTNSQSD LDSIAQETQR	129	100		1 Mascot	
2	phase 1 flagellin [Salmonella enterica]	gi 38050067	51510	4.84	1083	100	8				
Peptide Information											
	Calc. Mass	Obsrv. Mass	± da	± ppm	Start Seq.	End Sequence	Ion Score	C. I. %	Modification	Rank Result Type	

1132.5957	1132.5677	-0.028	-25	21	31	SQSALGT ^X IER	75	100	1 Mascot
1285.7111	1285.6799	-0.0312	-24	412	423	IDAALAQVDTLR	109	100	1 Mascot
1614.8195	1614.7792	-0.0403	-25	38	53	INSAKDDAAGQAIANR	170	100	1 Mascot
2007.0254	2006.9684	-0.057	-28	433	451	FNSAITNLGNTVNNLTSA R	160	100	1 Mascot
2805.3496	2805.2722	-0.0774	-28	94	119	ELAVGSANSTNSQSDLD SIQAEITQR	204	100	1 Mascot
2860.6003	2860.511	-0.0893	-31	469	495	AQILQQAGTSVLAQANQ VPGNVL ^S LLR	180	100	1 Mascot
3060.5193	3060.4424	-0.0769	-25	92	119	VRELAVQSANSTNSQSD LDSIQAEITQR	129	100	1 Mascot

Gel Idx/Pos		12/A12	Instr./Gel Origin		ak167/6616-083112		Process Status		Analysis Succeeded		
Plate [#] Name		[1] 6616-083112		Instrument Sample Name				Spectra		13	
Rank	Protein Name		Accession No.	Protein MW	Protein PI	Total Ion Score	Total Ion C. I. %	Pep. Species Count	Molecular Function		
1	flagellin [alternatively spliced] [Salmonella typhimurium, SJW46, Peptide Mutant, 397 aa]		gj834004	41725.2	4.98	883	100	7			
Peptide Information											
	Calc. Mass	Obsrv. Mass	± da	± ppm	Start Seq.	End Sequence Seq.	Ion Score	C. I. %	Modification	Rank Result Type	
	1132.5957	1132.5653	-0.0304	-27	20	30 QSALGTAIER	52	99.989		1 Mascot	
	1285.7111	1285.6749	-0.0362	-28	314	325 IDAALAQVDTLR	109	100		1 Mascot	
	2007.0254	2006.9608	-0.0646	-32	335	353 FNSAITNLGNTVNNLTSA _R	162	100		1 Mascot	
	2670.3076	2670.2131	-0.0945	-35	66	90 NANDGISIAQTTEGALNEI _{NNLQR}	170	100		1 Mascot	
	2805.3496	2805.2463	-0.1033	-37	93	118 ELAVQSANSTNSQSOLD _{SIQAEITQR}	190	100		1 Mascot	
	2860.6003	2860.4934	-0.1069	-37	371	397 AQILQOAGTSVLAQANQ _{VPQNVLSLLR}	104	100		1 Mascot	
	3060.5193	3060.4067	-0.1126	-37	91	118 VRELAVQSANSTNSQSOLD _{LDSIQAEITQR}	96	100		1 Mascot	
2	phase 1 flagellin [Salmonella enterica]		gj38050067	51510	4.84	883	100	7			
Peptide Information											
	Calc. Mass	Obsrv. Mass	± da	± ppm	Start Seq.	End Sequence Seq.	Ion Score	C. I. %	Modification	Rank Result Type	
	1132.5957	1132.5653	-0.0304	-27	21	31 QSALGTXIER	52	99.989		1 Mascot	
	1285.7111	1285.6749	-0.0362	-28	412	423 IDAALAQVDTLR	109	100		1 Mascot	
	2007.0254	2006.9608	-0.0646	-32	433	451 FNSAITNLGNTVNNLTSA _R	162	100		1 Mascot	
	2670.3076	2670.2131	-0.0945	-35	67	91 NANDGISIAQTTEGALNEI _{NNLQR}	170	100		1 Mascot	
	2805.3496	2805.2463	-0.1033	-37	94	119 ELAVQSANSTNSQSOLD _{SIQAEITQR}	190	100		1 Mascot	
	2860.6003	2860.4934	-0.1069	-37	469	495 AQILQOAGTSVLAQANQ _{VPQNVLSLLR}	104	100		1 Mascot	
	3060.5193	3060.4067	-0.1126	-37	92	119 VRELAVQSANSTNSQSOLD _{LDSIQAEITQR}	96	100		1 Mascot	